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FRANCESCO LOTTI

**TRANSCRIPTIONAL TARGETING OF LENTIVIRAL
VECTORS TO THE ERYTHROBLASTIC PROGENY OF
HEMATOPOIETIC STEM CELLS**

**A THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS OF THE
OPEN UNIVERSITY FOR THE DEGREE OF DOCTOR OF PHILOSOPHY**

APRIL 2003

DIBIT

**Department of Biological and Technical Research,
Istituto Scientifico Ospedale San Raffaele
Milan, Italy**

Declaration

This thesis has been composed by myself and has not been used in any previous application for a degree. The results presented here were obtained by myself.

All sources of information are acknowledged by means of reference.

Part of this work has been published in *J. Virol.*, 2002 (see reference Lotti *et al.*, 2002) and has been orally presented at the 5th Annual Meeting of the American Society of Gene Therapy (ASGT) and at the 10th Annual Meeting of the European Society of Gene Therapy (ESGT).

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All this work is dedicated to the memory of Emilio Menguzzato.

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Abstract

Correction of blood genetic disorders requires permanent gene transfer into self-renewing, hematopoietic stem cells (HSC), and regulation of transgene expression in specific cell lineages. HIV-derived lentiviral vectors are very effective in transducing rare, non-dividing stem cell populations without altering their long-term repopulation and differentiation capacity. We developed a strategy for transcriptional targeting of lentiviral vectors based on replacing the viral LTR control elements with cell lineage-specific, genomic control elements. An upstream enhancer (HS2) of the erythroid-specific GATA-1 gene was cloned in a second-generation lentiviral vector to replace most of the U3 region of the LTR, immediately upstream of the HIV-1 promoter. The modified LTR was used to drive the expression of a reporter gene (GFP), while a second gene (Δ LNFR) was placed under the control of an internal, constitutive promoter to monitor cell transduction, or immunoselect transduced cells, independently from the expression of the targeted promoter. The vector was used to transduce cell lines, human CD34⁺ hematopoietic stem/progenitor cells, and murine bone marrow HSCs. Gene expression was analyzed in the differentiated progeny of transduced stem cells *in vitro* and *in vivo*, after transplantation into lethally irradiated co-isogenic (for murine cells) or NOD/SCID (for human cells) mice. The transcriptionally targeted HIV LTR allowed very high level of transgene expression specifically in mature erythroblasts, in a *tat*-independent fashion and with no alteration in titer, infectivity, and genomic stability of the lentiviral vector. Expression from the targeted LTR was higher, better restricted, and showed significantly less position effect variegation than that obtained by the same combination of enhancer/promoter elements placed in the conventional, internal position. Cloning of the woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) at a defined position in the targeted vector, allowed selective accumulation of the genomic with respect to the internal RNA transcript, with no loss of cell-type restriction. A critical advantage of this targeting strategy is the use of the spliced, major viral transcript to express a therapeutic gene, and that of an internal, independently regulated promoter to express an additional gene for either cell marking or *in vivo* selection purposes.

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Erythroid Kruppel-like factor (EKLF)

The Fli-1 oncogene

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Abbreviations used in this thesis (unless otherwise stated in the text)

<i>Kb</i>	Kilobases
Bp	Base pairs
RNA	Ribonucleic acid
DNA	Deoxyribonucleic acid
Gp	Glycoprotein
IL-#	Interleukin-number
HIV	Human Immunodeficiency Virus
ECM	Extracellular matrix
NOD/SCID	Non-Obese diabetic/Severe combined immunodeficiency
LTR	Long Terminal Repeat
SCF	Stem Cell Factor
TPO	Trombopoietin
WPRE	Woodchuck post-transcriptional
cPPT	Central polypurine tract
Epo	Erythropoietin

Chapter 1

INTRODUCTION

1.1 Haematopoiesis: an overview.

All the cells that circulate in the peripheral blood are derived from primitive mesenchymal cells referred to as pluripotential hematopoietic stem cells (HSCs). In the adult, most of these cells are located in the bone marrow, but they are capable of circulating freely in the peripheral blood. Indeed, small numbers of these cells are always present in the peripheral bloodstream in transit from one site in the marrow to another via hematogenous spread; in this manner, the marrow is continuously “seeded” with these cells in a homogenous fashion. In the marrow of a healthy person, most stem cells are neither dividing nor differentiating. These cells are considered to be in a prolonged intermitotic interval and comprise the reserve stem cell pool that can be called into division during times of hematopoietic stress. Another smaller fraction of the total hematopoietic population is composed of cells that are dividing but not differentiating. Finally, at any given moment in time, a relatively tiny portion of the total stem cell pool is made up of cells that are beginning to differentiate along one the several pathways of hematopoietic development through a process that ultimately gives rise to the wide diversity of cells that circulate in the peripheral blood. This elegant process, which generally is restricted to the bone marrow, is referred to as haematopoiesis (Fig. 1.1).

In vertebrates, the site of hematopoiesis shifts during development from the yolk sac site to the fetal liver, then to the bone marrow in the perinatal period (Fig. 1.2). Primitive haematopoiesis, that is associated with the production of large nucleated embryonic erythroblasts, occurs in blood islands that emerge from extraembryonic mesoderm in the yolk sac, at murine embryonic day 7.5 (E7.5) or day 15-18 in humans (Orkin and Zon, 1997). Definitive hematopoiesis, characterised by well-defined enucleated erythrocytes first takes place in the fetal liver beginning at mouse E9.5; it is multilineage, generating myeloid, megakaryocyte and lymphoid cells (Dzierzak and Medvinsky, 1995). It is generally believed to initiate in the aorto-gonad-mesonephros (AGM) region (Dzierzak et al., 1998), though a more recent study suggests that the yolk sac is the predominant source of both primitive and definitive hematopoietic progenitors (Palis et al., 1999). Hematopoietic progenitors migrate through the blood stream to seed the fetal liver. Late in fetal life, bone marrow assumes hematopoietic activity and becomes the predominant hematopoietic organ in postnatal life (Medvinsky and Dzierzak, 1996) (Fig.1.2).

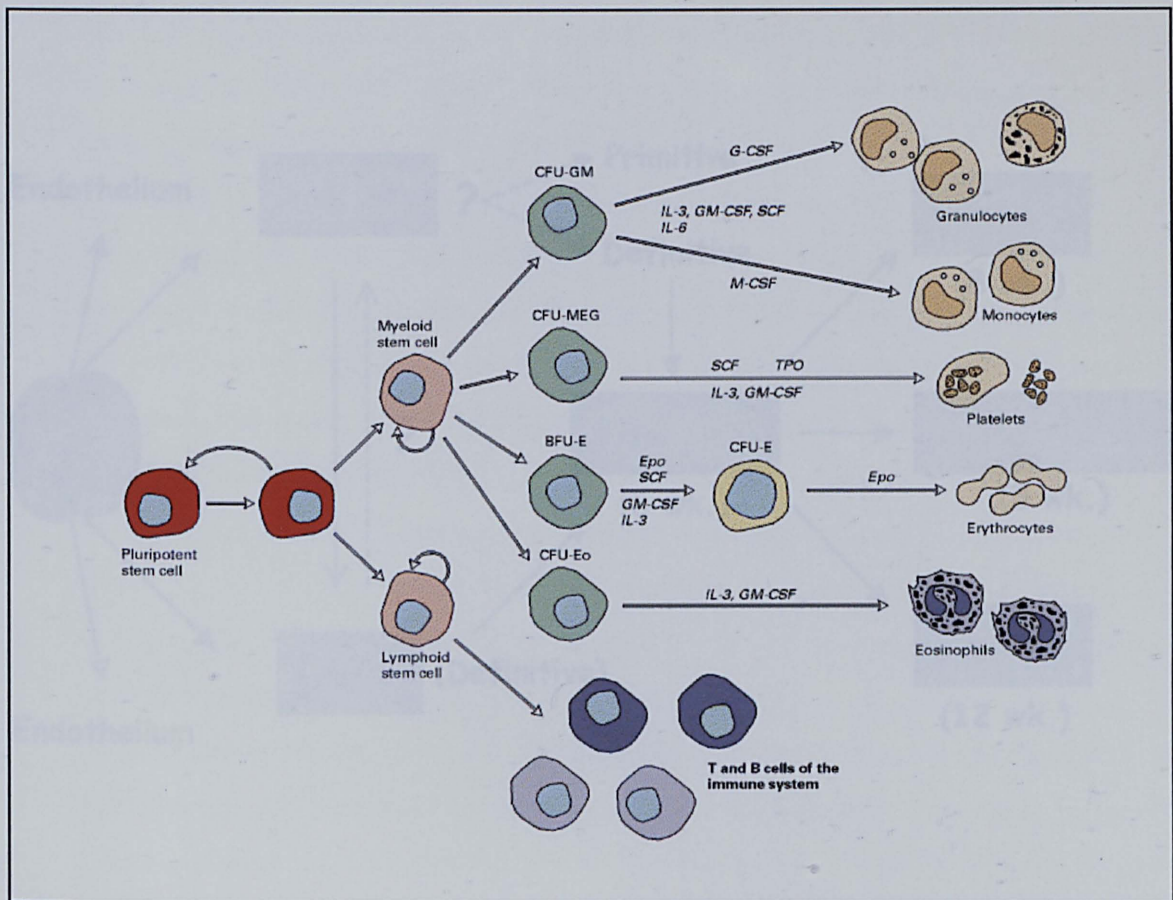


Figure 1.1. Haematopoiesis. This illustration describes the development of mature blood cells through the sequential restriction of the cell fate potential of oligopotent progenitor cells derived from multipotent HSC.

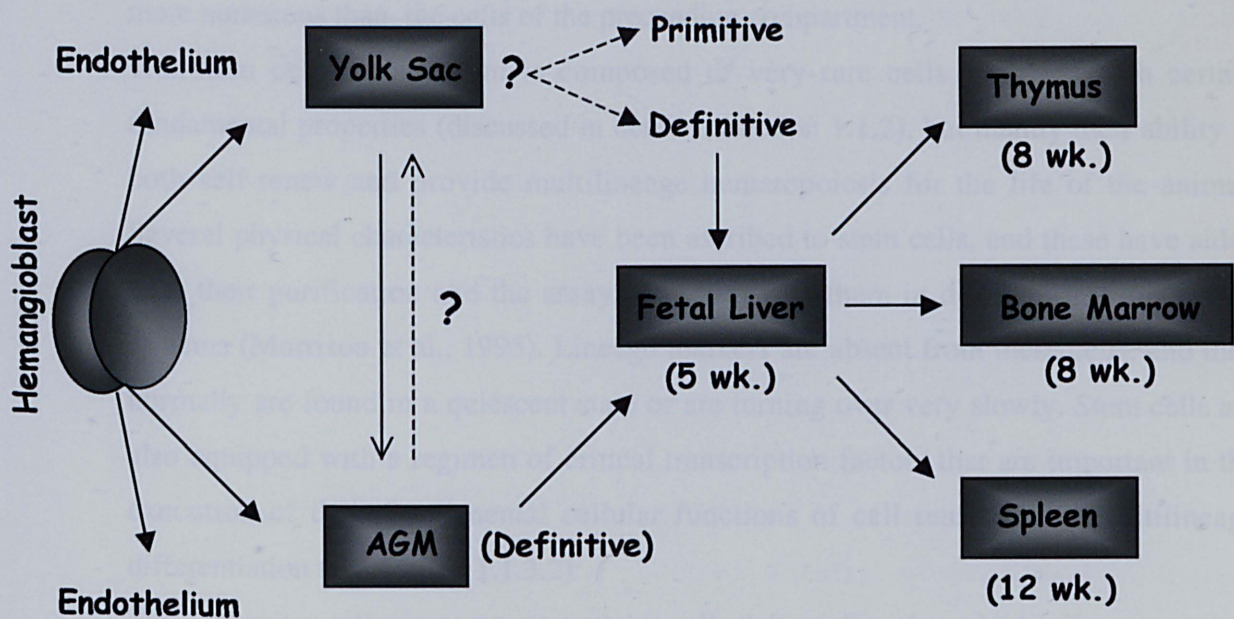


Figure 1.2. Models of ontogeny of mammalian haematopoiesis. The two main sources of haematopoietic progenitor cells are the yolk sac and the AGM region. At day 9, the yolk sac contributes an early line of CFU-C cells that probably does not last long after birth, and which makes a population of predominantly red blood cells. This cell population is thought to be the major source of the first wave of haematopoiesis in the liver. At day 10, the AGM-derived cells provide CFU-S cells and pluripotential haematopoietic stem cells. These constitute the major cells of the second wave. Definitive progenitor cells present in circulation colonize the liver at 5-6 weeks and full development of liver haematopoiesis is established by 8-9 weeks and maintained until about 12 weeks, when bone marrow and spleen are colonized. Bone marrow is the exclusive site of haematopoiesis in the adult animal.

1.1.1 Organization of Hematopoiesis: cell compartments

Hematopoietic cells can be broadly classified as transiting through three compartments: (1) stem cell compartment, (2) committed progenitor cells, and (3) mature functional-end cells (Fig. 1.1). The cells in each succeeding compartment are the progeny of, and more numerous than, the cells of the preceding compartment.

The stem cell compartment is composed of very rare cells endowed with certain fundamental properties (discussed in detail in section 1.1.2), but mainly their ability to both self-renew and provide multilineage hematopoiesis for the life of the animal. Several physical characteristics have been ascribed to stem cells, and these have aided both their purification and the assay used to define them in different transplantation systems (Morrison et al., 1995). Lineage markers are absent from these cells, and they normally are found in a quiescent state or are turning over very slowly. Stem cells are also equipped with a regimen of critical transcription factors that are important in the execution of their fundamental cellular functions of cell renewal and multilineage differentiation (see section 1.1.3.2).

The progenitor cell compartment contains cells that are found at a higher frequency than the stem cell pool and, like the stem cell, are not morphologically distinguishable. Their existence is revealed by their ability to give rise to differentiated progeny *in vitro* in well-defined functional assays (see section 1.1.2.2). The progenitor cell compartment is derived from stem cells through a process of commitment to different lineage pathways. Transition of stem cells to cells of the committed compartment is achieved not by acquisition of new characteristics or new proteins but by enhancement of certain molecular pathways, already primed in these cells, and abrogation of others (Hu et al., 1997). Within each lineage, a spectrum of progenitor cells exists, and these are hierarchically categorized on the basis of their proliferative potential, maturation time, response to a set of cytokines and type of differentiated progeny as revealed through *in vitro* clonogenic cultures. For example, multilineage progenitors giving rise to multiple lineages *in vitro* (e. g., colony-forming unit- granulocytic, erythroid, eosinophil, macrophage, megakaryocyte [CFU-GEMM]) are presumed to be more primitive than unilineage progenitors committed only to a single lineage.

As progenitor cells differentiate, they acquire more distinctive features characteristic of each lineage and move away from shared primitive progenitor characteristics: they show the enhancement of lineage-specific features, with a diminished or absence of expression of multilineage properties. Progenitor cells of each lineage are internally

programmed to ensure their own survival, but external cues are frequently impinged on these internal program that control differentiation, maturation, survival and migration (Wagers et al., 2002) (see section 1.1.3).

The precursor cell compartment, in contrast to stem and progenitor compartments, is defined by morphological criteria and contains cells at different maturation stages; the cells may be capable of undergoing a few rounds of cell division, or they may be end-stage, non-mitotic cells with a finite life span. The morphological characteristics of these cells reflect the accumulation of lineage-specific proteins, and organelles and the decline of nuclear activity, which gives them a unique appearance. Furthermore, precursor cells for each lineage follow a unique maturation sequence; for example, in erythroid cells, the end product is an enucleated red cell, as the nuclei are expelled before terminal maturation. By contrast, terminally mature white cells remain nucleated. Also, cells of megakaryocytic lineage undergo unique endoreduplication cycles, forming large cells with multilobulated nuclei. The maturation sequence for each lineage requires a specific time frame, but there is enough plasticity to allow for faster than normal production of end-stage effector cells. Such deviations from the normal sequence are dictated by stress, which demands quick delivery of specialized (mainly white) cells into the periphery.

1.1.2 The Hematopoietic stem cell

Hematopoietic stem cells (HSCs) are probably the best characterized stem cell population. The properties that define these cells are: (i), they have the capacity to generate differentiated progeny of multiple blood cell lineages; (ii), they have the potential to produce more stem cells through a process known as self-renewal. It is this capacity to self-renew that enables the stem cell population to sustain hematopoiesis over extended periods of time (perhaps indefinitely) *in vivo* and, in fact, distinguishes them from virtually all other cells within the hematopoietic system (Abruzzese et al., 1999; Jordan and Lemischka, 1990; Keller and Snodgrass, 1990; Morrison et al., 1996). The theory of HSCs was first put forward in 1964 (Till *et al*, 1964), but to date it has not been comprehensively identified and isolated. Indeed, the only means of assaying stem cells is by their ability to reconstitute the bone marrow of an irradiated host and give rise to the hematopoietic system (radioprotection assay). This has been demonstrated primarily in humans undergoing bone marrow transplantation and also in experimental animal models, where the endogenous hematopoietic system was ablated by a lethal dose of radiation (Aguila et al., 1997). The frequency of HSCs in the bone marrow is argued to be 1×10^{-4} to 1×10^{-5} (Murray et al., 1994). Due to its rarity, a robust effort has been dedicated to enrich HSCs to allow further characterization studies. HSCs have been enriched using a varieties of techniques, including density centrifugation, activation and/or cell-cycle status and surface antigen expression, but no unique characteristics have been found to identify these elusive cells specifically. A crucial point in the isolation of the HSCs is the one-to-one correspondence between physically purified cells and their potential ability to function as stem cells (see below).

1.1.2.1 Phenotypic characterization of HSC

Although the history of the application of BM transplants to humans involved the use of a large number of animal pre-clinical models, including the dog, pig and non-human primates, many of the relevant experiments leading to the isolation and characterization of HSC were derived from studies using mice. Thus, particular attention has been dedicated to principles of HSC isolation, characterization and transplantation in the mouse, providing correlative experiments in human.

Properties of mouse HSC/progenitor subsets

Limiting dilution reconstitution experiments in congenic mice using inbred strains that differed only at the CD45 molecule by two known alleles, CD45.1 and CD45.2, allowed the isolation of at least two classes of multipotent stem cells: long term (LT-HSCs) and short term (ST-HSCs) reconstitutive cells (Morrison and Weissman, 1994; Smith et al., 1991; Uchida et al., 1997). The long-term subset self-renews for the entire lifespan of the host, while the short-term subset retained self-renewal capacity for approximately 8 weeks. A third, more abundant and less completely characterized population of multipotent progenitors (MPPs) include multipotent cells of limited productive lifespan and, probably, without any self-renewal (Morrison and Weissman, 1994). The three classes of cells are arranged in a lineage from LT-HSCs to ST-HSCs, which in turn give rise to MPPs (Fig. 1.3) (Morrison et al., 1997). Each stage of differentiation of multipotent cells involves functionally irreversible maturation steps. ST-HSCs and MPPs do not produce LT-HSCs, and MPPs do not produce ST-HSCs. Included in the progeny of mouse HSCs are two kinds of oligolineage-restricted cells: common lymphocyte progenitors (CLPs), which at a clonal level are restricted to give rise to T lymphocytes, B lymphocytes, and Natural Killer (NK) cells (Kondo et al., 1997) and CMPs, which are progenitors for the myeloerythroid lineages (Akashi et al., 1999). CMPs give rise to myelomonocytic progenitors (GMPs) and megakaryotic/erythroid progenitors (MEPs). All of these populations (LT-HSCs, ST-HSCs, MPPs, CLPs, CMPs, GMPs and MEPs) are separable as pure populations using cell surface markers (Fig. 1.3) (Akashi et al., 1999; Weissman, 2000). The total population of mouse multipotent progenitors is contained within cells that are c-kit⁺ Thy1.1^{lo} Lin⁻/lo Sca-1⁺ (KTLS cells) (Spangrude et al., 1988). The Lin designation is for markers found on the surface of blood cells of known committed lineages, such as T cells, B cells, granulocytes, monocytes, erythrocytes and natural killer cells. Of the Lin markers, two are important for the separation of the different multipotent progenitor populations: Mac-1 and CD4. The most primitive population of LT-HSCs are contained in the Mac-1⁻ and CD4⁻ subset of KTLS cells, while their immediate descendants, the ST-HSCs, are Mac-1^{low} and CD4⁻. The other population of MPPs is characterized by a Mac-1^{low} and CD4^{low} phenotype (Morrison and Weissman, 1994). Common lymphocyte progenitors (CLPs) has been found to be represented at about 0.02% of mouse BM and can be isolated with the phenotype Lin⁻ IL7R⁺ Thy-1⁻ Sca-1^{-lo} c-kit^{-lo} (Fig. 1.3).

In 1996, Goodell et al. reported a new method of obtaining enriched populations of HSCs from adult mouse bone marrow (Goodell et al., 1996). This procedure exploits the ability of HSCs to efflux the fluorescent dye, which like the activity of P-glycoprotein (encoded by the MDR gene), is verapamil-sensitive. The Hoechst 33342 low cells thus isolated were called the side population (SP) cells and were found to have the same Lin⁻ Scal⁺ CD34⁻ phenotype independently identified in adult murine HSCs (Yusa and Tsuruo, 1989). SP cells have since been identified in adult bone marrow from several species including humans (Miller and Eaves, 1997).

Properties of human HSC/progenitor subsets

As with isolation of mouse HSC, human HSC and progenitor isolation has been characterized by enriching a rare cell population with a combination of monoclonal antibodies. Over the past decade, various cell surface and metabolic markers have been identified and used to isolate human HSC and progenitors. Among them, CD34, originally identified by the My 10 monoclonal antibody (Civin et al., 1984), became the most critical cell surface marker to define the phenotype of human HSCs. For most experimental purposes purification of CD34 high expressing cells from peripheral blood (0.1% of total cells), bone marrow (1-3% of total cells), or umbilical cord blood (0.3-1% of total cells), in association with low granularity and/or low levels of CD45 expression, can reliably give rise to a population of CD34⁺ cells that include HSCs. However, CD34 expression is not restricted to the most early hematopoietic progenitors but is also present on many of the intermediate cells, between the most primitive hematopoietic cells to more committed progenitors. Furthermore, several groups provided evidence of various types of human HSCs that do not express detectable levels of CD34 (Zanjani et al., 1998). Therefore, intense research has focused on identifying other characteristics, in either association or not with CD34 expression, which may serve as more reliable indicators of the HSC. The most widely used associated antigen is CD38. This is typically a marker for thymocytes, but is also found on plasma cells, and activated T-lymphocytes. Cells that do not express, or express low levels of this antigen in association with high levels of CD34 (CD34⁺CD38^{-/lo}), constitute a widely accepted phenotype for identification of primitive HSC. During differentiation, the cells up-regulate expression of CD38, therefore serving as a useful marker to distinguish between CD34⁺ primitive HSC and CD34⁺ progenitor HSC. Other associated antigens that can be used to identify primitive HSC include the type 2 major histocompatibility

antigen, HLA-DR, which is absent from the most primitive HSC, but is acquired during stem cell ontogenesis. Another intriguing marker for primitive HSC is Thy-1. More than 25% of hematopoietic cells express this marker, but if combined with CD34 expression, it is argued that a cell expressing both these antigens at high levels represents a very rare and early HSC (Chen et al., 1997).

In conclusion, it is clear that the HSC has a wide range of phenotypes, however, the CD34⁺Thy-1⁺ Lin⁻ designation identifies human HSC in fetal liver and BM, umbilical cord blood, adult bone marrow and mobilized peripheral blood (Baum et al., 1992; Murray et al., 1995).

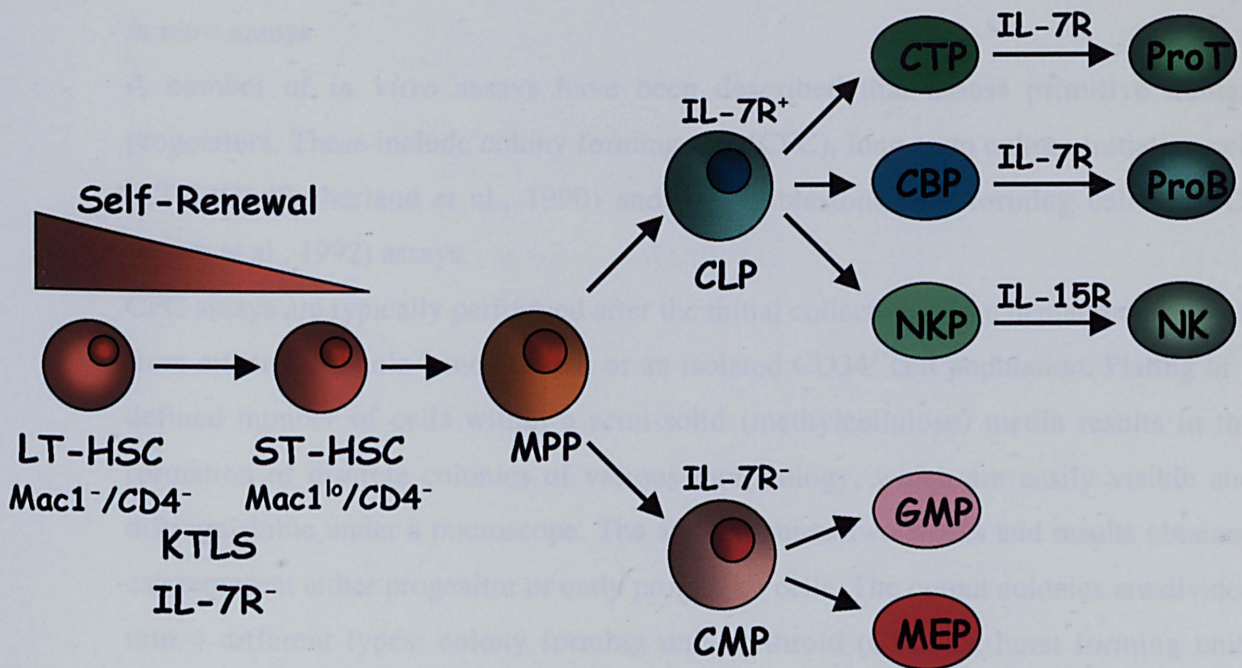


Figure 1.3. Model of HSC differentiation. Included in the progeny of mouse HSCs are two kinds of oligolineage-restricted cells: common lymphocyte progenitors (CLPs), which can be isolated with the phenotype Lin⁻IL-7R⁺Thy1.1⁻Sca-1^{lo}c-Kit^{lo}, and which at the clonal level are restricted to give rise to T lymphocytes, B lymphocytes, and natural killer (NK) cells; and CMPs, which are progenitors for the myeloerythroid lineages. CMPs give rise to cells that include myelomonocytic progenitors (GMPs) and megakaryotic/erythroid progenitors (MEPs). All of these populations are separable as pure populations using cell surface markers (adapted from Weissman, Cell 2000).

1.1.2.2 Assays for the HSC

Human HSC functional activity can be analyzed quantitatively by *in vitro* and *in vivo* assays.

In vitro assays

A number of *in vitro* assays have been described that assess primitive human progenitors. These include colony forming cell (CFC), long-term culture initiating cell (LTC-IC) (Sutherland et al., 1990) and the cobblestone area-forming cell (CAFC) (Baum et al., 1992) assays.

CFC assays are typically performed after the initial collection of the hematopoietic cells from either the whole bone marrow or an isolated CD34⁺ cell population. Plating of a defined number of cells within a semi-solid (methylcellulose) media results in the formation of discrete colonies of various morphology, which are easily visible and distinguishable under a microscope. The assay requires two weeks and results obtained can represent either progenitor or early progenitor cells. The output colonies are divided into 4 different types: colony forming unit-erythroid (CFU-E), burst forming unit-erythroid (BFU-E), colony forming unit-granulocyte/macrophage (CFU-GM) and colony forming unit- granulocyte/erythroid/macrophage/megakaryocyte (CFU-GEMM or CFU-MIX).

LTC-IC assays utilise bone marrow stromal monolayers to support the maintenance and/or growth of primitive hematopoietic progenitors over at least 5 weeks (Sutherland et al., 1990). Only primitive cells can be maintained over this time period and once plated in methylcellulose, following the CFC-based assay, will give rise to colonies.

The CAFC assay is very similar to the LTC-IC assay (Baum et al., 1992). The plating of HSC on preformed stromal monolayers results in the formation of small groups of cells attached to the stroma, which are visible after 28-32 days.

These assays enumerate primitive myeloid progenitors, but not cells with multi-lineage differentiation or self-renewal potential. More recently, several groups have developed cultures that allow the differentiation of single human Lin-CD34⁺ cells into cells with myeloid, natural killer (NK), B-lymphoid, dendritic, and/or T-lymphoid phenotypes, showing that a single cell can differentiate *in vitro* into multiple lineages (Hao et al., 1998; Miller et al., 1999). However, all of these assays have some limitation. First of all, none of these assays are able to generate secondary primitive progenitors that again have multilineage differentiation potential, thus arguing that they select for certain

populations, raising doubts as to whether it is the HSC is in fact being selected. Secondly, there is a poor correlation between results obtained *in vitro* and the results obtained *in vivo*.

In vivo assays

Demonstrations of hematopoietic activity *in vivo* are much more revealing, but much more difficult to perform, than *in vitro* assays. Most assays involve the use of murine animal models but assays involving sheep and non-human primates have also been used to demonstrate the long-term repopulating behaviour of these cells (Donahue et al., 1996; Dunbar et al., 1996; Ziegler et al., 1999).

An *in vivo* model for human HSC/progenitor cell engraftment was developed using xenogenic transplantation of human cells into immunodeficient mice. The two main types of immunodeficient mice are *bnx* (beige/nude/xid), which lack both T and NK cells (Nolta et al., 1996) and SCID, which lack functional T and B cells (Kamel-Reid and Dick, 1988). In the SCID-hu bone marrow model, candidates for human HSC populations can be examined for their ability to reconstitute allogeneic human fetal bone giving rise to B-, myeloid and CD34⁺ progenitor cells (Kyoizumi et al., 1992; McCune et al., 1988). The T-cell potential of such candidate populations can be characterised in the SCID-hu thymus model (Peault et al., 1991). Human HSC/progenitors can engraft and differentiate into myeloid and CD3⁺ cells in *bnx* mouse BM (Nolta et al., 1996).

Most of the earlier studies used SCID mice, which were not ideal as they still possessed significant antigen-non-specific immunity. As a result, high cell doses were required to overcome any residual host resistance, ruling out the development of quantitative assays and any purification strategies. A new mouse strain, generated by crossing SCID into the non-obese diabetic (NOD) background, proved to be a better recipient. Compared with SCID mice, this new NOD/SCID strain appears to have lower NK and complement activities and a defect in macrophage function. A lower number of cells (10 to 20-fold less) were necessary to engraft NOD/SCID mice when compared with SCID mice. Overall, these mice showed high levels of engraftment for normal and leukaemic human transplants and, more importantly, enabled engraftment with lower cell doses, rendering purification strategies possible (Laroche et al., 1996). The only limitation of these NOD/SCID mice is their inability to support human T-cell development. On the contrary, beige-nude-SCID (Bnx) mice allow T-cell development, but not B-cell differentiation. Other strains have also become available recently: β 2-microglobulin

knockout/NOD/SCID, Rag1 knockout/NLD, and the nude/NOD/SCID mice. The capabilities of each of these new strains still need to be explored.

1.1.2.3 Correlation of phenotype and function

Although CD34⁺ cells represent a large section of the hematopoietic microenvironment, they have been demonstrated to possess reconstitution potential (Peault et al., 1991). CD34⁺ cells injected into a SCID-hu (thymus model) mouse gives rise to engraftment and generation of CD3, CD4, CD8 and T cells. More importantly, this population of cells was also shown to repopulate baboons. Additionally, they are routinely used for humans, thus confirming their potential. However, whether they represent long-term or short-term repopulating cells is still not clear.

CD34⁺ Thy-1⁺ Lin⁻, CD34⁺ Thy-1⁺, CD34⁺ HLA-DR⁻ and CD34⁺ CD38⁺ CD33⁺ cells have all been demonstrated to reconstitute mice and sheep. The CD34⁺/HLA-DR⁻ cell population was demonstrated to reconstitute sheep, giving rise to virtually all the hematopoietic lineages over at least 7 months. The CD34⁺/Thy-1⁺ cell population is routinely used for SCID and SCID-hu experiments, giving rise to both myeloid and B-lymphoid cells. The CD34⁺ CD38⁺ CD33⁺ cell population has obtained popularity for reliably reconstituting NOD/SCID mice (Laroche et al., 1996). However, all of these phenotypes are rare, and it is difficult to plan extensive experiments employing these cell types, due to the difficulty and low efficiency in obtaining them.

The most widely used phenotype for animal experiments, believed to represent the repopulating stem cell, is the CD34⁺/CD38⁻ cell, which has been used extensively for the reconstitution of mice (reviewed in Verfaillie et al., 2002).

1.1.3 Molecular control of Hematopoiesis

The complex orchestration of hematopoiesis through which the elaborate array of blood cells described above is produced requires three physiological components, each of which is essential. These are: (1) the stem cell pool itself; (2) hematopoietic cytokines, that regulate hematopoiesis through both endocrine and paracrine mechanisms; and (3) the hematopoietic inductive microenvironment, which is made up of the bone marrow stroma and vasculature. In addition to these extracellular cues, it is evident that critical steps in the control of HSC fate, from the very earliest switching of mesenchymal cells to the hematopoietic lineage to differentiation into the various lineages, are transcriptionally regulated.

Most studies of HSC fate determination focus on the choice between stem cell self-renewal or differentiation. Over the years, several models have been advanced proposing that hematopoietic lineage determination is driven extrinsically (through growth factors, stroma or other external influences), intrinsically (as described in stochastic models), or both. While several studies support an instructive role for cytokines in the maturation of terminally differentiated cells from progenitor and precursor cells (Kaushansky et al., 1995; Kondo et al., 2000; Metcalf and Burgess, 1982) the initial divisions of HSC are likely to involve stochastic fate choices (Ogawa, 1993; Ogawa, 1999). In fact, it has been postulated that stochastic determination of HSC cell fate may be important for HSC function, in that this scheme allows for the maintenance of production of all blood cell lineages even in the face of substantial demand for one particular lineage (Enver and Greaves, 1998). Supporting the stochastic model for HSC differentiation, *in vitro* culture of Bcl-2 transgenic HSC with stem cell factor (SCF) alone induces these cells to proliferate, but does not preserve their primitive status (Domen and Weissman, 2000). This induction of HSC differentiation in the absence of any overt differentiating signals, suggests that lineage choice need not be initiated by particular cytokine signals. Similarly, lineage-restricted progenitors can develop from HSC at normal frequency, even in the absence of lineage-specific cytokines (Kondo et al., 1997).

Overall, it appears that the regulation of hematopoiesis is the result of multiple processes involving cell-cell and cell-extracellular matrix interactions, the action of specific growth factors and other cytokines, as well as intrinsic modulators of hematopoietic development.

1.1.3.1 Role of the microenvironment in HSC fate determination

Regulation of HSC fate involves multiple highly orchestrated pathways that determine cell cycle status and gene expression profile. This complexity is due in large part to the fact that HSCs do not grow as independent autonomous units. Rather, these cells are surrounded in all dimensions by the marrow microenvironment, the so-called “stem cell niche”. The hypothetical function of the HSC niche is to provide HSC survival and self-renewal factors, either through direct contact with HSC or through secreted factors. HSC niches are formed from a subset of mesenchymal cells within the bone marrow. These so-called “stromal cells”, which include specialized fibroblasts, endothelial cells, osteoblasts, and perhaps adipocytes, may play a further role in “translating” external signals to influence HSC developmental decisions (Muller-Sieburg and Deryugina, 1995).

HSC are exposed *in situ* to many different growth factors, some soluble, some bound to extracellular matrix (ECM) and others bound to adjacent cells. Due to the inherent difficulty in studying these cells in their microenvironment, most of what we know about how different soluble growth factors control HSC fate has been deduced from *in vitro* studies (Ema et al., 2000; Goff et al., 1998). The primary question that has been addressed is whether we can simulate *in vitro* the conditions that tell the HSC to self-renew. If this were completely understood and could be performed *in vitro*, we could then expand small numbers of highly pluripotent HSC for clinical transplantation as well as for other scientific studies. Due to their fundamental role in mediating *in vitro* maintenance and expansion of stem cells, growth factors and cytokines will be described in detail in Section 1.1.3.3.

Within the context of a supportive microenvironment, cell adhesion and extracellular matrix molecules also play an important role in HSC development. Within the adult bone marrow, these molecules mediate interactions between stromal and hematopoietic cell components that in turn regulate cell proliferation, differentiation and migration. Extracellular matrix (ECM) is composed of three major classes of molecules: structural proteins such as collagen and elastin, specialized proteins such as fibronectin and laminin, and proteoglycans which consist of a protein core to which is attached long chains of repeating disaccharide units, termed glycosaminoglycans (GAGs). ECM proteins in the BM include fibronectin, collagen, laminin and cytokines, which can bind to ECM proteins. The ECM acts in concert with cell-cell interactions and soluble factors to regulate the HSC. In general, adhesion of hematopoietic stem and progenitor cells to

the marrow ECM inhibits cellular proliferation and prevents apoptosis, both of which lead to long-term survival of quiescent hematopoietic stem cells. Clues are beginning to emerge regarding how these effects are mediated. For example, binding of integrins on hematopoietic stem or progenitor cells has been shown to lead to increased p27 expression, and p27 halts progression of the cell cycle by inhibiting a cyclin dependent kinase (Cheng et al., 2000).

1.1.3.2 Transcriptional regulation of HSC fate

Transcription factors represent a nodal point of hematopoietic control through the integration of the various signaling pathways and subsequent modulation of the transcriptional machinery. Transcription factors can act both positively and negatively to regulate the expression of a wide range of haematopoiesis-relevant genes including growth factors and their receptors, other transcription factors, as well as various molecules important for the function of developing cells. It is the alternative expression of specific combinations of transcription factors that determines the survival, proliferation, commitment, and differentiation responses of hematopoietic progenitors to such signals, whether they arise from extrinsic or intrinsic regulatory factors. Increasing evidence suggests that different families of transcription factors regulate the developmental program of stem cells (Shivdasani and Orkin, 1996) and when their expression is disrupted, leukaemic proliferation is initiated (Rabbitts, 1994).

Since purifying and biochemically investigating very rare HSC populations is so difficult, knowledge of the role of specific transcription factors in HSC fate decisions has been derived largely from genetic strategies, primarily gene-targeting (knockout) and retroviral infection/overexpression experiments. From this growing body of literature, several transcription factors have been found to play critical roles in HSC physiology, and some of them are described in the sections below depending on the time and the influence of their expression during different steps of HSC development.

1.1.3.2.1 Transcription factors required during early stem cell decisions

Lying at the top of a regulatory hierarchy would appear to be those genes that are required for the formation of all hematopoietic lineages. Such genes might act at the pre-HSC level, for example, within mesoderm or the hemangioblast, to specify a hematopoietic fate or within the HSC to foster survival or proliferation. Genes in this general class include the transcription factors known as SCL/Tal-1, GATA-2, and LMO-2, which are essential for primitive and definitive hematopoiesis, and AML-1 that is required for definitive hematopoiesis (Fig. 1.4).

SCL/Tal-1 protein shares homology within a restricted region of 56 amino acids with a number of bHLH transcription factors, and it is capable of binding to the E-box motif of DNA (CANNTG) *in vitro* and forms heterodimers with other HLH factors such as E47/E2A and E12. SCL homozygous null embryos die at around 9.5 d.p.c. and histological studies showed complete absence of recognizable hematopoiesis in the yolk sac, with GATA-1 and c-Myb expression being undetectable (Robb et al., 1995). Very recently, the group of Orkin reported that SCL/Tal-1 is dispensable for HSC engraftment, self-renewal and differentiation into myeloid and lymphoid lineages, while proper differentiation of erythroid and megakaryocytic precursors is dependent on SCL/Tal-1 (Mikkola et al., 2003). Thus, SCL/Tal-1 is essential for the genesis of HSCs, but its continued expression is not essential for HSC function.

Both primitive hematopoiesis in the yolk sac and definitive hematopoiesis in fetal liver and spleen as well as adult bone marrow are greatly affected by deficiency in GATA-2. Deficient primitive haematopoiesis, evident by severe anemia, results in most GATA-2-deficient embryos dying before 10.5 p (Tsai et al., 1994). GATA-2 may also be one major mediator of the ability of BMP-4 to specify hematopoietic mesoderm, since BMP-4 expression in the embryo is polarized to the ventral wall of the aorta, immediately underlying the site of initial hematopoiesis (Maeno et al., 1996; Marshall et al., 2000).

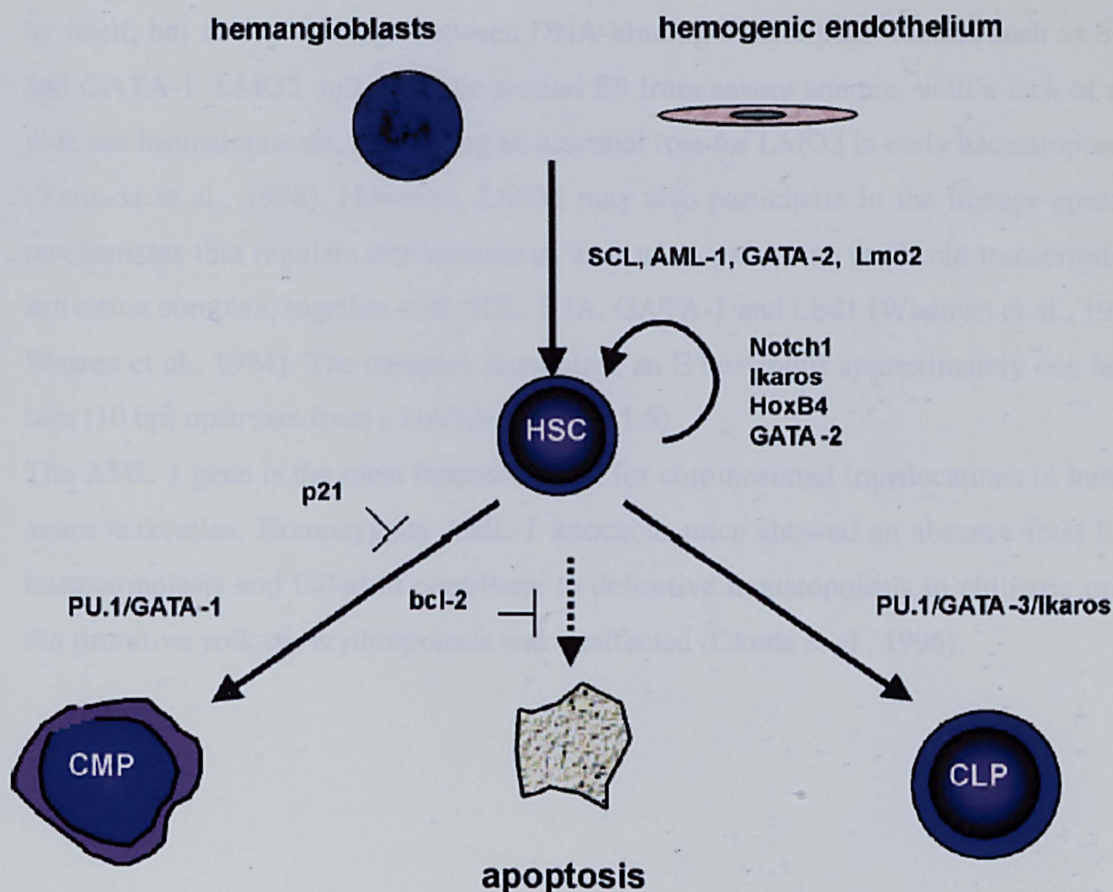


Figure 1.4. Transcriptional regulation of early HSC development. HSCs, as one progeny of hemangioblasts or hemogenic endothelium, are faced with the cell fate choice either to self-renew or to differentiate into committed common lymphoid or common myeloid haematopoietic precursors. The transcription factors involved in each development direction are depicted (see text for details. Adapted from Zhu J and Emerson SG, *Oncogene*, 2002).

Lim-finger protein LMO2, also known as Rbtn2, was originally found to be activated in T cell leukemia by chromosomal translocation. Embryogenic LMO2 expression was found localized to hematopoietic sites (Manaia et al., 2000). LMO2 does not bind DNA by itself, but acts as a bridge between DNA-binding transcription factors such as SCL and GATA-1. LMO2 null mice die around E9 from severe anemia, with a lack of any yolk sac haematopoiesis, identifying an essential role for LMO2 in early haematopoiesis (Yamada et al., 1998). However, LMO2 may also participate in the lineage-specific mechanisms that regulate erythropoiesis, as it takes part in an erythroid transcription-activation complex, together with SCL, E2A, GATA-1 and Lbd1 (Wadman et al., 1997; Warren et al., 1994). The complex recognizes an E box motif approximately one helix turn (10 bp) upstream from a GATA site (Fig. 1.5).

The AML-1 gene is the most frequent target for chromosomal translocations in human acute leukemias. Homozygous AML-1 knockout mice showed an absence fetal liver haematopoiesis and failed to contribute to definitive hematopoiesis in chimeric mice, but primitive yolk sac erythropoiesis was unaffected (Okuda et al., 1996).

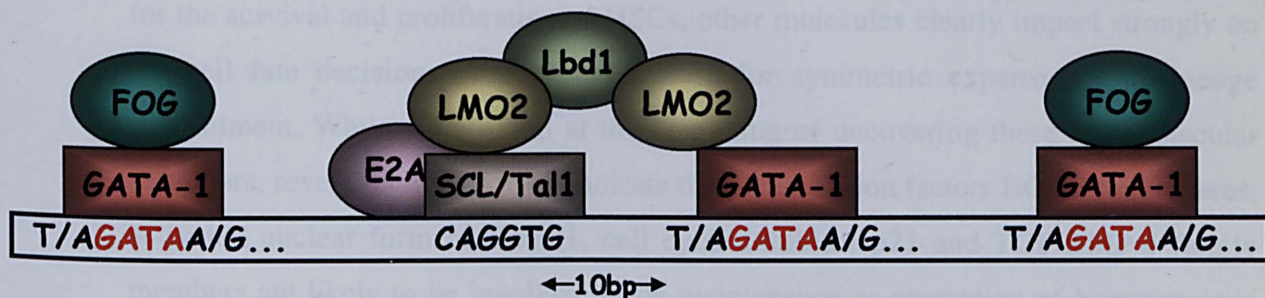


Figure 1.5. Pentameric complex formed between SCL, E2A, LMO2, Ldb1 and GATA-1. schematic representation of cis-acting regulatory DNA from a hypothetical erythroid target gene containing an E box motif (CAGGTG) and GATA-binding motif separated by 10 nucleotides (adapted from Wadman *et al.*, 1997).

1.1.3.2.2 Transcription factors regulating self-renewal of HSCs

The transcriptional machinery governing primitive stem cell biology is undoubtedly very complex. While the transcription factors described above are absolutely essential for the survival and proliferation of HSCs, other molecules clearly impact strongly on the cell fate decisions of stem cells, both for symmetric expansion and lineage commitment. While we are still at the beginning of uncovering these key molecular regulators, several pieces of work indicate that transcription factors HOXB4 and Ikaros, activated nuclear form of Notch1, cell cycle inhibitor p21 and TGF/BMP-4 family members are likely to be involved in the maintenance or promotion of hematopoietic stem cell renewal (Fig. 1.4).

Homeobox (HOX) gene family members encode DNA-binding transcription factors characterized by a conserved 60-amino acid homeodomain which is homologous to *Drosophila* homeobox proteins and also plays a crucial role in mammalian embryonic axis formation. Human HOX genes are organized on different chromosomes in four major clusters, A, B, C and D, each of which consists of nine to 12 tandem genes (Scott, 1992). While their role in embryonic axis formation has been well studied, their potential function in regulating haematopoiesis is less clear, although several recent studies point to a major role (Sauvageau et al., 1994). For example, it was first noticed that *in vitro* differentiation of human Lin⁻, non-adherent peripheral blood cells to either the erythroid or granulocytic fate was accompanied by a remarkable and persistent increase in HoxB4 mRNA levels, and that the administration of HoxB4 antisense oligonucleotides inhibited colony formation to these two lineages *in vitro* (Giampaolo et al., 1994). Then it was shown that the long-term repopulating ability of murine bone marrow cells was increased by at least 10-fold when HoxB4 cDNA was overexpressed by retroviral infection (Sauvageau et al., 1995; Thorsteinsdottir et al., 1999). More recently, the same group also reported that Hox B4 is able to induce *ex vivo* expansion of adult HSCs (Antonchuk et al., 2002).

The Ikaros gene product belongs to the zinc finger family of transcription factors. The long form of Ikaros mainly consists of two zinc fingers, an N-terminal finger domain mediating DNA binding and a C-terminal domain mediating dimerization. Generally, Ikaros proteins modulate transcription by recruiting a co-repressor complex to the promoters of target genes and/or sequestering these genes to the vicinity of heterochromatin (Koipally et al., 1999; Sabbattini et al., 2001; Trinh et al., 2001). Intriguingly, different isoforms of Ikaros are differentially expressed at different stages

of hematopoiesis (Klug et al., 1998), suggesting that regulated expression of Ikaros isoforms could provide fine regulation of the expression of lineage specific transcription factors. Mice lacking all isoforms of Ikaros display a severe deficiency in B, T and NK cell development (Georgopoulos et al., 1994).

In contrast to the transcription factors described above, which are believed to be directly regulated by only intracellular events, Notch1-Jagged may provide a key pathway to integrate extracellular regulatory signals with stem cell cycling control. Notch and jagged are members of a superfamily of highly evolutionarily conserved transmembrane receptors that influence numerous cell-fate decisions in both invertebrates and vertebrates. Members of both the Notch and Jagged superfamilies are predominantly transmembrane molecules. When a Notch family member binds to its ligand (a Jagged family member), a signal is transferred to cleave the intracellular domain of Notch, which then translocates to the nucleus where it interacts with cofactors to affect the transcriptional program. By constitutively overexpressing the intracellular domain of Notch1, several immortalized cell lines were established from murine BM SKL cells, which still maintained the potential to reconstitute myeloid and lymphoid cell lines both *in vivo* and *in vitro* (Varnum-Finney et al., 2000). This phenomenon may reflect the *in vivo* physiology of Notch/Jagged interactions, as the addition of Notch ligand jagged-1 into an *in vitro* expansion system of human CD34⁺ CD38⁻ Lin⁻ cord blood cells, prompted their ability to repopulate pluripotently in SCID mice (Karanu et al., 2000).

1.1.3.2.3 Transcriptional regulation of lineage commitment

Several transcription factors characterized to date exhibit restricted patterns of expression among the maturing precursor cells and are specifically required for development of one or more hematopoietic lineages. Some of these factors act in very different lineage decisions, such as PU.1 and Notch1 and their influence on HSC fate may vary depending on the time and the level of their expression during hematopoietic development (see Figs. 1.6 and 1.7).

Once HSCs divide and generate more differentiated daughter cells, within 10-15 divisions the genetic programs of the descendent cells become fixed toward a single lineage. How are lineage commitment decisions from HSCs initiated and regulated? This critical but most difficult question has been the subject of intense and creative debate and equally intense and creative experimentation. Among the most intriguing set of relevant data are those suggesting that HSC, CLP and CMP fate decisions might not

be instantly made, but rather preceded by a phase of promiscuousness or hesitation (Enver and Greaves, 1998; Rothenberg, 2000). The alternative differentiation potential might not be immediately eliminated, but rather repressed in a graded or gradual way in the cells that are committing to a given lineage.

Presumably, the differential expression of transcription factors triggers the determination of HSC fate: renewal or commitment to either CLP or CMP. By using differential cDNA library screening and sensitive RT-PCR methods, a number of candidate transcription factors that are preferentially expressed in cells destined to one of those fates have been found. Of the transcription factors studied to date, the largest body of published evidence relates to the transcription factor PU.1. Predominance of PU.1 could be among one of earliest events biasing HSCs to lineage commitment. The co-upregulation of PU.1 and GATA-1 heralds the commitment to CMPs (see Fig. 1.6), PU.1 expression is maintained in CLPs and is also absolutely required for lymphoid development (see Fig. 1.7), while knock-out of PU.1 spared the development of primitive erythropoiesis (Scott et al., 1994).

Whereas PU.1 and GATA-1 are co-expressed in CMPs, their mutually exclusive expression coincides with further commitment to either granulocytic/monocytic or megakaryocytic/erythroid differentiation. The expression of GATA-1 decreases in granulocytic/monocytic differentiation while that of PU.1 decreases with differentiation to either megakaryocytic or erythroid direction (see Fig. 1.6). Moreover, gene targeting experiments have shown that PU.1 is absolutely required for monocytic and B lymphocytic development while GATA-1 for erythroid and megakaryocytic development. If hematopoietic lineage commitment is preceded by a state of transcriptional promiscuity, one may ask what is the mechanism that reinforces and stabilizes commitment to specific lineages. Studies suggest that the expression of a transcription factor, above a threshold level, has two reinforcing lineage directing effects: (1) auto-upregulation of itself (Chen et al., 1995; Tsai et al., 1991) and (2) down-regulation of alternative transcription factor pathways. In the clearest system studied to date, PU.1 was found to inhibit the transcriptional activity of GATA-1 upon its target genes and vice versa. The overexpression of PU.1 inhibited DNA binding of GATA-1 to its consensus element on target gene promoters and this inhibiting effect could be relieved by the over-expression of GATA-1 (Nerlov et al., 2000; Zhang et al., 1999). Taken together, these data suggest that the initial ratio of GATA-1/PU.1 protein at an early, critical time point in the CMP determines the subsequent lineage-restricted

fate of the cell (Cantor and Orkin, 2001; Rekhtman et al., 1999; Zhang et al., 1999; Zhang et al., 2000).

A sea of transcription factors regulating the subsequent lineage choice of the CMP and CLP has been identified. This is beyond the immediate scope of this study, but the reader is referred to any publication, by the guru in this field, S. Orkin (Cantor and Orkin, 2001; Shivdasani and Orkin, 1996).

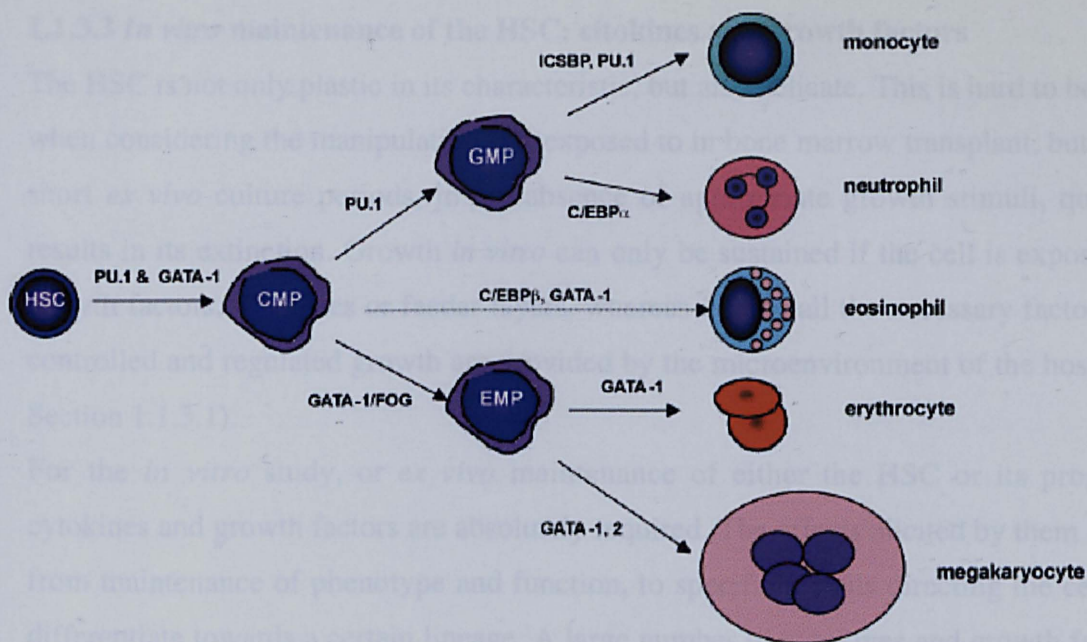


Figure 1.6. Transcriptional regulation of common myeloid precursor (CMP) commitment. CMPs differentiate into either common precursors for granulocytic and monocytic lineages (GMPs) or common precursors for both erythroid and megakaryocytic lineages (EMPs). A separate, possible, pathway leading to eosinophils is depicted by dotted line. Dual expression of PU.1 and GATA-1 leads HSCs to CMPs, but then dominant expression of PU.1 is restricted to GMPs, while unopposed GATA-1 expression directs differentiation to EMPs (from Zhu J and Emerson SG, Oncogene, 2002).

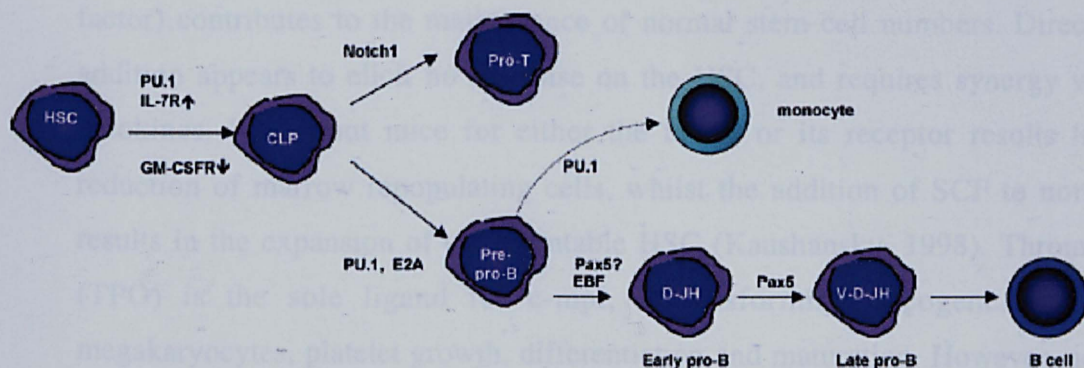


Figure 1.7. Transcription regulation of common lymphoid precursor (CLP) commitment. B lymphocytes and T lymphocytes are derived from a common lymphoid precursor (CLP). The early development of B cell is distinguished into distinct stages by the sequential expression of different transcription factors that direct Ig gene recombination and the expression of B cell specific cell surface phenotypes. A proposed differentiation pathway of macrophages from pro-B is also indicated by a dotted line (from Zhu J and Emerson SG, Oncogene, 2002).

1.1.3.3 *In vitro* maintenance of the HSC: cytokines and growth factors

The HSC is not only plastic in its characteristic, but also delicate. This is hard to believe when considering the manipulation it is exposed to in bone marrow transplant, but even short *ex vivo* culture periods, in the absence of appropriate growth stimuli, quickly results in its extinction. Growth *in vitro* can only be sustained if the cell is exposed to growth factors, cytokines or feeder layers, whereas *in vivo* all the necessary factors for controlled and regulated growth are provided by the microenvironment of the host (see Section 1.1.3.1).

For the *in vitro* study, or *ex vivo* maintenance of either the HSC or its progeny, cytokines and growth factors are absolutely required. The effects elicited by them range from maintenance of phenotype and function, to specific signals directing the cells to differentiate towards a certain lineage. A large number of cytokines and growth factors regulate hematopoiesis, both in positive and negative ways, and this section is dedicated to the major cytokines that have been or are used *in vitro* to study and maintain the HSC (Alexander, 1998; Dexter and Heyworth, 1994).

Interleukin-3 (IL-3), also known as multi-CSF, is a multilineage stimulator, directly giving rise to megakaryocytes, mast-cell/basophils, B-cells and eosinophils. Interleukin-6 (IL-6) has a direct proliferative effect on hematopoietic cells, including the growth of both B and T cells, but in synergy with other factors can induce myeloid cell proliferation. If used with IL-3, it can induce the proliferation of primitive hematopoietic progenitors. Stem cell factor (SCF, also known as Kit-ligand and Steel factor) contributes to the maintenance of normal stem cell numbers. Direct *in vitro* addition appears to elicit no response on the HSC, and requires synergy with other cytokines. Knock-out mice for either the factor or its receptor results in a large reduction of marrow repopulating cells, whilst the addition of SCF to normal mice results in the expansion of transplantable HSC (Kaushansky, 1998). Thrombopoietin (TPO) is the sole ligand for c-mpl, a transforming oncogene. It stimulates megakaryocytes, platelet growth, differentiation and maturation. However, addition to umbilical cord blood derived HSC can result in expansion of the HSC pool and also expansion of LTC-IC in serum free culture (Kaushansky, 1998). However, the same effect has not been observed for mobilised peripheral blood derived CD34⁺ cells (Murray et al., 1999), but has been observed for bone marrow derived CD34⁺/Thy⁺/Lin⁻ cells (Young et al., 1996). Flt-3 is a class III tyrosine kinase receptor, expressed almost exclusively on human CD34⁺ HSC. Flt-3 Ligand can recruit primitive CD34⁺/CD38⁻

HSC into cycle to a greater extent than combination of cytokines (Haylock et al., 1997), although the receptor expression is low on the most primitive HSC, but higher on intermediates between it and committed progenitors.

Very few cytokines act alone, most of them synergistically with other cytokines, to produce an effect. Any combination of different cytokines can result in the growth and maintenance of the HSC, however more specific approaches to determine which cytokines are best for the *ex vivo* maintenance of *in vivo* repopulating cell are constantly being tested. The use of IL-3, IL-6 and SCF was a standard for HSC culture and has been used in combination or substitution with other colony stimulating factors during *in vitro* culture. The culture of CD34⁺/CD38⁻ cells for 3 weeks in SCF, TPO, Flt3-L give rise to higher levels of engraftment compared to a TPO, Flt3-L, IL-6 supplemented culture. The addition of IL-6 to the SCF, TPO, Flt3-L culture combination gave no benefit, indicating that SCF was absolutely required, for *ex vivo* maintenance of the repopulating HSC.

Studies analysing a large variety of cytokine combinations or cytokines alone demonstrated that the induction of cycling, and maintenance of the CD34⁺ phenotype did not vary greatly between the combinations used (Murray et al., 1999). Prevention of apoptosis, the percent of cell viability and the percent of primitive phenotype were better controlled using the SCF, TPO, Flt3-L alone or in combination, compared to combinations containing LIF, IL-3, or IL-6 (Murray et al., 1999). However, an important role of HSC and hematopoietic control is attributed to its environment and particularly to the stroma.

1.1.4 The Hematopoietic System as a Target for Gene Therapy

Gene therapy employs various methods to deliver foreign genes into somatic cells with the ultimate goal of incorporation and stable expression of the gene of interest in a manner which will result in a therapeutic effect. Methods used for gene therapy are categorized into non-viral and viral. Non-viral methods of gene delivery include cationic and cholesterol-containing liposomes, peptide lipid vectors, activated dendrimers, bacteria, artificial chromosomes and artificial viruses. Most of the current gene therapy approaches make use of the viral methods. The viral vectors used for gene transfer include retroviruses, adenoviruses, adeno-associated viruses, herpes simplex virus and flaviviruses. For gene therapy to be successful, an appropriate amount of a therapeutic gene must be delivered into the target tissue without substantial toxicity. Each viral vector system is characterized by an inherent set of properties that affect its suitability for specific gene therapy applications. For some disorders, long-term expression from a relatively small proportion of cells would be sufficient (for example, genetic disorders), whereas other pathologies might require high, but transient, gene expression. In other cases, such as diabetes and thalassemias, gene therapy will require tightly regulated gene expression (Clackson, 2000). No single vector system is likely to be optimal for all the potential gene therapy applications. However, for a specific application, an “ideal” vector will be administered by non-invasive delivery routes, target to the desired number of cells within target tissue and express a therapeutic amount of transgene product with the desired regulation for a defined length of time. Though we are likely to see gene therapy successes in the near future, the true fruition of gene therapy cannot be realized until the current vectors are perfected or new vectors developed with the properties described above.

Over the last two decades, it has been proposed that the transfer of genes into hematopoietic stem cells could be a tool for the treatment of genetically inherited diseases as well as a number of acquired diseases, including cancer, AIDS, autoimmune and neurodegenerative disorders. With the clinical application of gene therapy progressing in parallel with a better understanding of stem cell biology, the number of different target cells, tissues and organs used in gene therapy has increased significantly. However, HSCs remain the main cellular target for genetic intervention in a number of clinical settings of primary medical and scientific relevance, especially, but not only, those that aim to correct or modulate the immune system.

1.1.4.1 Viral vectors for gene transfer into HSC

All viral and synthetic vectors developed for different gene therapy models have been tested for their ability to transduce HSCs with most of the data being generated with viral vectors, predominantly murine onco-retroviruses (Kay et al., 2001). These vectors permanently integrate the transferred gene into the genome of the host cell, which should maintain transgene expression during proliferation, differentiation and maturation in all cell lineages. However, target cell division is required for gene transduction with these vectors, whereas immature HSCs are naturally quiescent. To overcome this initial obstacle, a large body of research focused on inducing cell division while preserving the self-renewal ability of stem cells and their potential to expand and differentiate into all blood lineages. Strategies involved the use of different hematopoietic growth factors in various combinations, with or without the additional use of bone marrow stromal cell layers and human recombinant fibronectin fragments. The abilities of these strategies to favour gene transfer efficiency while preserving "stem cell potential" of the transduced population were explored. This massive effort resulted in significant progress in the development of animal models of HSC gene transfer. However, for several years this research had no impact on gene therapy for human diseases. Data on the feasibility of stem cell gene therapy in humans only became available a few years ago in a small number of clinical settings, including severe-combined immunodeficiencies (SCIDs) and several gene-marking studies in the context of bone marrow transplantation (Bordignon et al., 1995; Kohn, 1999). But either the clinical relevance of these results was difficult to evaluate or transgene expression was below the threshold required for clinical benefit. Only very recently, a clear clinical benefit has been observed in the treatment of two different types of SCID diseases (Aiuti et al., 2002); Cavazzana-Calvo et al., 2000). However, in both these cases the relevance of the outcome was partially attributed to the selective advantage that the transduced cells shared with respect to the untransduced cells.

The discovery and development of new vectors derived from HIV sharply accelerated progress in the field of HSC gene transfer. This family of lentiviral vectors can transduce dividing and nondividing cells from different lineages, tissues and organs, including HSCs, with great efficiency (Naldini et al., 1996). Lentiviral vectors efficiently transduce nondividing cells after direct *in vivo* injection, thus making genetic engineering easier and more suitable for large-scale medical application. However, safety concerns related to the use of vectors derived from a human pathogen and technical problems associated with large-scale production and validation have hindered their application as a new gene transfer tool.

1.2 Lentiviruses, HIV-based vectors and lentiviral packaging cell system

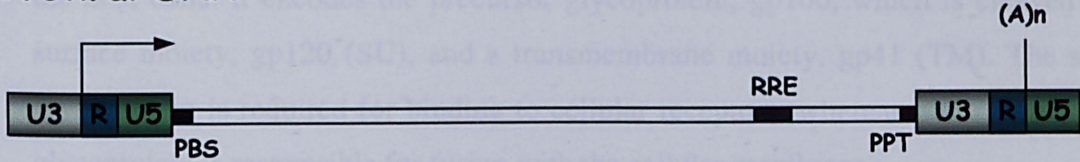
Lentiviruses are a family of complex retroviruses typically associated with infection of macrophages and lymphocytes. Lentiviruses include a variety of primate (e.g. human immunodeficiency viruses [HIV-1 and 2], and simian immunodeficiency viruses [SIV]) and non-primate (e.g. maedi-visna virus [MVV], feline immunodeficiency virus [FIV], equine infectious anemia virus [EIAV], caprine arthritis encephalitis virus [CAEV] and bovine immunodeficiency virus [BIV]) viruses (Coffin et al., 1997). The ability to integrate into the genome of non-dividing cells makes lentiviruses particularly attractive in human gene therapy.

1.2.1 Lentiviral genome structure and vector elements

The lentiviral genome is a diploid, plus stranded RNA that is reverse transcribed into DNA (called a provirus) and integrated into the host cell chromosomes (Fig. 1.8). Like other retroviruses, the provirus contains the long terminal repeat (LTR) elements at both 5' and 3' ends. The LTRs contain the left and right integration attachment sites (att) and are required for integration into the host genome. The LTRs also serve as enhancer-promoter sequences, controlling expression of the viral genes. Each LTR contains U3 (unique 3), R (repeat), and U5 (unique 5) regions. The U3 contains the core promoter and adjacent regulatory sequences, both of which recruit the cellular transcriptional machinery to the start site of viral RNA synthesis. The R region contains the sequence for the trans-acting response (TAR) element and signals for polyadenylation. The U5 region contains sequences that contribute to the packaging of progeny RNA genomes during virus assembling.

HIV-1 encodes three structural genes: gag, pol and env. The product of gag is translated from unspliced mRNA as a precursor protein and cleaved into the following protein subunits: matrix (MA), essential for virion assembly and infection of non-dividing cells (Gallay et al., 1996; Gallay et al., 1995) capsid (CA), which forms the hydrophobic core of the virion and is essential for virion assembly and maturation; nucleocapsid (NC), which coats viral RNA stoichiometrically and remains tightly associated with viral RNA in virions; p6 which is important for Vpr incorporation during viral assembly (Selig et al., 1999) and for the efficient particle release (Huang et al., 1995). The pol gene encodes three enzymes required for viral replication: protease (PRO), reverse transcriptase (RT) and integrase (IN). PRO cleaves Gag and Gag-Pol polyproteins which are essential for the maturation of the virion. RT contains three enzymatic

Proviral DNA



Genomic RNA

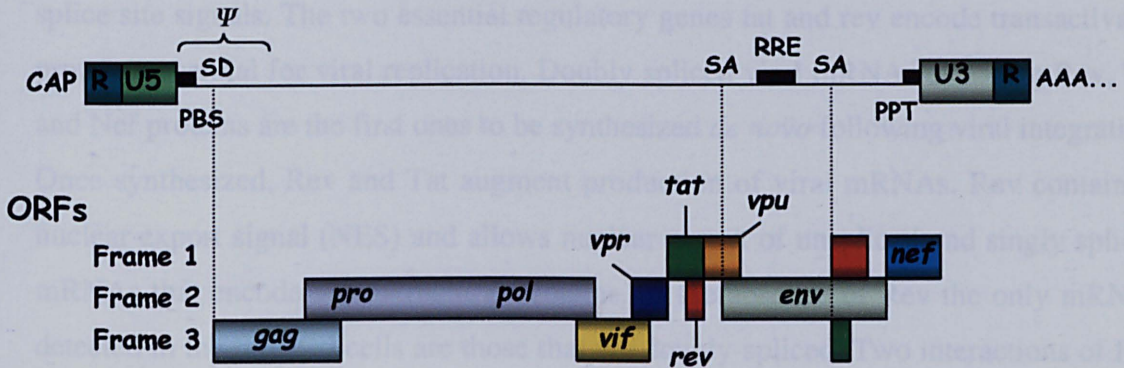


Figure 1.8. Genetic organization of generalized provirus. The proviral DNA as it is inserted into host DNA is shown at the top, with the long terminal repeats (LTRs) composed of U3, R, and U5 elements at each end abutting cellular sequences. Sequences in the LTR that are important for transcription, for example, enhancers, the promoter, and the poly(A) addition signal, are marked. The gag, pro, pol, and env sequences are located invariably in the positions shown in all retroviruses. Accessory genes are located as shown, and also overlapping env and U3 and each other, and occasionally in other locations. The RNA that is the primary transcriptional product is shown on the second line. Sequences that are important for replication and gene expression are shown in the approximate locations in which they are typically found. (PBS) Primer-binding site; (Ψ) encapsidation sequence; (SD) splice donor site; (SA) splice acceptor site; (PPT) polypurine tract; (A)ⁿ polyadenylation signal; (AAA...) poly(A) tail.

activities: RNA-dependent DNA polymerase, RNAase H and DNA-dependent DNA polymerase. The env gene is essential for viral binding and entry into the host cells. It encodes the precursor glycoprotein, gp160, which is cleaved into a surface moiety, gp120 (SU), and a transmembrane moiety, gp41 (TM). The surface glycoprotein is required for binding to cellular receptors, whereas the transmembrane glycoprotein is responsible for fusion with the cellular membrane.

All lentiviral regulatory and accessory proteins are encoded by variously spliced viral mRNAs that are generated from this full-length viral transcript using different 5' and 3' splice site signals. The two essential regulatory genes tat and rev encode transactivator proteins essential for viral replication. Doubly spliced viral mRNAs encoding Rev, Tat and Nef proteins are the first ones to be synthesized *de novo* following viral integration. Once synthesized, Rev and Tat augment production of viral mRNAs. Rev contains a nuclear export signal (NES) and allows nuclear export of unspliced and singly spliced mRNAs that encode viral structural proteins. In the absence of Rev the only mRNAs detected in the infected cells are those that are doubly-spliced. Two interactions of Rev are required for attaining this function. First, Rev interacts with the Rev Responsive Element (RRE) overlapping the env coding sequence. Second, Rev interacts with the nuclear pore proteins, nucleoporins. Rev, therefore, acts as a shuttle between the nucleus and cytoplasm, mediating the nuclear export of the RRE-containing viral mRNAs. The viral protein Tat upregulates viral transcription at the level of elongation via interaction with the Tat activation region (TAR) located at the 5' end of all viral mRNAs. In addition to interacting with TAR, the binding to the cyclin-dependent kinase-9 (CDK-9)-Cyclin T1 complex has also been shown to be required for the activity of Tat (Wei et al., 1998). Tat interacts through its activation domain (AD) with the cyclin T1 subunit of a preexisting TAK (Tat-associates Kinase) complex. The interaction of Tat with the cyclin T1 alters the conformation of Tat to enhance greatly the affinity and specificity of the Tat:TAR interaction. Subsequent to the binding to TAR, CDK-9 phosphorylates the C-terminal domain (CTD) of RNA polymerase II ensuing an efficient elongation of the viral transcription (Kim et al., 2002).

The accessory genes include vif, vpr, vpu and nef. These genes were named accessory because they are nonessential for virus replication in cell culture (Luciw, 1996). Vif (virion infectivity factor) is not essential for HIV-1 replication in permissive cells such as HeLa-CD4 or SupT1 (Gabuzda et al., 1992; Sakai et al., 1993). However, it is necessary for production of infectious virions by cells that are natural targets for

infection, including CD4-positive T-lymphocytes, macrophages, and H9 cells (Fouchier et al., 1996; von Schwedler et al., 1993). A recent study has suggested that nonpermissive cells contain an endogenous inhibitor of HIV-1 production that is overcome by the virus-encoded Vif protein (Madani and Kabat, 1998). The potential role of Vif in an HIV-1-derived vector was directly examined and it was concluded that vif is dispensable (Kim et al., 1998; Zufferey et al., 1997). It may be inferred that the vector producing cells utilized in these studies may have a "permissive" phenotype with respect to Vif, and that the presence of Vif in a replication-defective vector may not be necessary because target cells will not produce viral progeny. Vpr (viral protein R) is a virion-associated protein present only in primate lentiviruses. The first function of Vpr to be documented was its ability to act as a weak transcriptional transactivator of the viral LTR (Ogawa et al., 1989). In addition, HIV-1 Vpr participates in viral infection of non-dividing cells (Balotta et al., 1993; Bukrinsky and Haffar, 1997; Heinzinger et al., 1994). Vpr has also been shown to cause cell cycle block in G2 and apoptosis (Jowett et al., 1995; Poon et al., 1998; Stewart et al., 1997). Despite its role in the transduction of non-dividing cells, vpr is not required in an HIV-1 packaging construct (Kim et al., 1998; Zufferey et al., 1997), due largely to the existence of the other mechanisms (MA and IN) allowing the nuclear transport of pre-integration complexes. The vpu gene encodes a cytoplasmic viral protein and is present exclusively in HIV-1. It promotes degradation of CD4 in the endoplasmic reticulum of target cells. Degradation of CD4 allows transport of Env to the cell surface and its incorporation into virions. Vpu is able to stimulate the release of viral particles from certain types of cells including T-lymphocytes, HeLa and colonic carcinoma SW480 cells (Klimkait et al., 1990). Another function of Vpu is to downregulate the expression of MHC I molecules on the surface of infected cells (Kerkau et al., 1997). This prevents recognition by cytotoxic T-cells. Despite its multiple functions, vpu is a dispensable gene in a vector system as its exclusion does not seem to influence the properties of the vpu-negative viral particles (Kim et al., 1998; Zufferey et al., 1997). The nef gene is only found in primate lentiviruses and it is essential for viral infectivity *in vivo*, but not *in vitro* (Kestler et al., 1991). Nef reduces interactions between Env and intracellular CD4 by inducing internalization and degradation of CD4 (Piguet et al., 1999). Nef also downregulates cell surface expression of MHC I molecules and protects infected cells from killing by cytotoxic T-lymphocytes (Collins et al., 1998). Nef was also shown to enhance the infectivity of viral particles, independently of the effects on CD4 (Goldsmith et al.,

1995). This Nef-dependent enhancement of infectivity occurs at the level of proviral DNA synthesis, early in the infectious cycle (Aiken and Trono, 1995). However, later studies demonstrated that the dependence on Nef for achieving optimal infectivity in a vector system could be overcome by the use of VSV-G glycoprotein-pseudotypes (Aiken, 1997). This observation suggested that Nef only increases infectivity of virions entering the cells by direct fusion with the cellular membrane, but not via receptor-mediated endocytosis. These results are in agreement with other studies which directly demonstrate that Nef is entirely dispensable in lentiviral vectors when pseudotyped with VSV-G (Zufferey et al., 1997).

1.2.2 Life cycle of Lentiviruses

The lentiviral life cycle is similar to that of all members of the Retroviridae family and consists of different steps as depicted in Fig 1.9.

Attachment and entry

The interaction of the lentivirus with the target cell occurs via binding of the viral envelope glycoprotein to a specific receptor on the cell plasma membrane, which defines the cellular target for the virus (viral tropism). Primate lentiviruses infect helper T-lymphocytes, macrophages, microglial, dendritic and Langerhans cells by interacting with CD4 and one of several chemokine receptors, most frequently CCR5 or CXCR4 (Bell, 1998; Zaitseva et al., 1997). Once bound to the cellular receptor, the viral membrane undergoes fusion with the cellular membrane. After virion-bound matrix and capsid proteins disassemble and the nucleoprotein complex is delivered into the cells, reverse transcription begins in the cytoplasm.

Reverse transcription

Reverse transcription leads to the synthesis of double-stranded, linear DNA from a single-stranded RNA template using cellular tRNA as a primer. The enzyme that catalyzes this step in viral replication, reverse transcriptase, has low fidelity due to the lack of proofreading ability and therefore, is partly responsible for the high variability of the viral genome. Recent studies have shown that during reverse transcription, a central DNA flap is created due to the presence of a central polypurine tract (cPPT) and central termination sequence (CTS) within the pol coding sequence (Charneau et al., 1992; Charneau et al., 1994). The creation of this unique triple-stranded DNA region appears to be important for the nuclear import of preintegration complex (PIC), in both dividing and non-dividing cells (Follenzi et al., 2000; Zennou et al., 2000). Recent vector designs have, therefore, incorporated the cis signals (cPPT and CTS) for the creation of this central DNA flap and thereby allow more efficient nuclear import of the vector genome.

Integration

Once synthesis of the linear viral DNA is complete, viral integrase performs specific cleavages at the 5' and 3' termini and catalyzes integration into the host genome. Integration is essential for retroviral gene expression (Sakai et al., 1993) and allows the provirus to become a permanent genetic element in the host.

Transcription and viral protein synthesis

Early transcription from the provirus results in the production of doubly spliced mRNAs encoding Rev, Tat and Nef. Translation of mRNA leads to accumulation of Tat and Rev. Together, Tat and Rev induce a shift to a "late" transcription mode whereby unspliced and singly spliced RNA species are primarily produced. Unspliced and singly spliced mRNAs encode structural genes necessary for the production of viral progeny.

Virion assembly and release

The viral RNA and structural proteins are packaged into viral particles and released by budding at the plasma membrane. After the polyproteins, Gag and Gag/Pol, are cleaved by the viral protease, mature particles become fully infectious. In currently available lentiviral vectors, gag/pol are not present in the transfer construct, but are provided in *trans* by a packaging construct (Naldini et al., 1996). Thus, once the transfer vector is integrated in the host cell, its inability to direct production of gag/pol ensures that there will be no subsequent viral progeny.

1.2.3 Lentiviral Vectors

Lentiviral (LV) vectors are replication-defective, hybrid viral particles made by the core

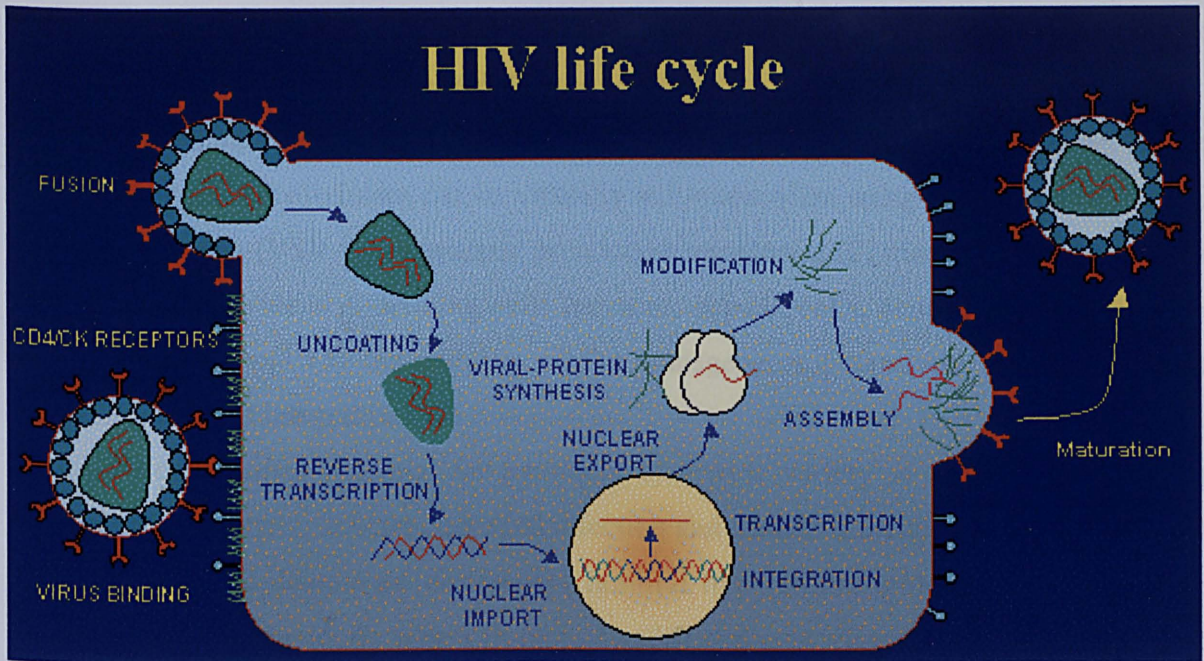


Figure 1.9. Lentiviral life cycle. Following binding of the retrovirus to its specific cell membrane receptor (CD4 and CK receptors), the viral and cellular membranes fuse, and the core virion is internalized into the cell. Reverse-transcriptase directed double-stranded retroviral genomic DNA is then generated, followed by integrase directed integration into host cell DNA. Retroviral transcripts using host transcriptional machinery then proceed, with the eventual formation of new retroviral virions that bud from the cell surface, allowing a new round of infection to occur.

1.2.3 Lentiviral Vectors

Lentiviral (LV) vectors are replication defective, hybrid viral particles made by the core proteins and enzymes of a lentivirus and the envelope of a different virus, most often the vesicular stomatitis virus (VSV).

The applicability of a safe lentiviral vector in human disease is broad because (1) the host range of lentiviruses can be virtually unlimited when using vesicular stomatitis G glycoprotein (VSV-G) to produce pseudotype envelopes; (2) many relevant targets for gene therapy are non-dividing cells (neurons, hepatic cells, hematopoietic stem cells, myocytes); and (3) the stability of the transgene is potentially indefinite due to chromosomal integration.

LV vectors offer potential for treatment of a wide variety of syndromes, including genetic/metabolic deficiencies, viral infection and cancer. Inherited genetic defects such as adenosine deaminase deficiency, familial hypercholesterolemia, cystic fibrosis, mucopolysaccharidosis type VII, types I and II diabetes, classical phenylketonuria and Gaucher's disease may be overcome by lentiviral vector-mediated gene therapy because they constitute single-gene deficiencies for which the involved genes are known.

Certain types of cancer may benefit from the use of LV vectors. Hypoxia and lack of vascularization lead to the generation of tumor cells which exhibit limited or no proliferation. Partly because of the lack of growth, these cells are highly resistant to genotoxic agents. A LV vector may prove to be a useful vehicle for delivery of a "lethal" gene (such as herpes virus thymidine kinase) to quiescent tumor cells.

Viral diseases may also constitute appropriate targets for lentiviral gene delivery. In particular, a number of gene therapy approaches have been proposed for the treatment of HIV infection. For some of these strategies, phase I studies have recently begun in humans. Preliminary studies have dealt with defective murine oncoviruses for delivery of anti-sense RNAs, RNAi, ribozymes and trans-dominant proteins against HIV replication.

1.2.3.1 Elements of a prototypical LV vector

The general strategies employed in the design of a LV vector involve segregation of trans-acting sequences that encode for viral proteins from cis-acting sequences involved in the transfer of the viral genome to target cells. The vector particles are assembled by viral proteins expressed in the producer cell from constructs stripped of the majority of viral cis-acting sequences. The viral cis-acting sequences are linked to the transgene and are introduced into the same cell. As the vector particle can only transfer the latter construct, the infection process is limited to a single round without further spreading.

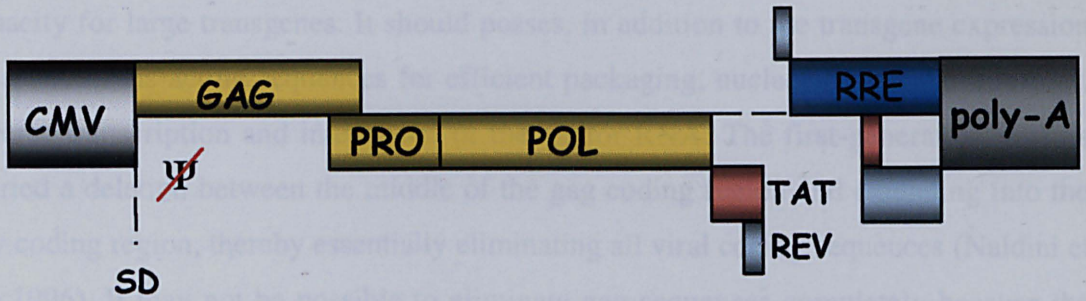
The packaging functions for the LV are provided by at least two separate expression plasmids that use transcriptional signals different from those of the virus (Fig. 1.10). A “core” packaging construct, derived from the HIV-1 proviral DNA, directs expression of viral structural proteins, except for the envelope. Proteins expressed by the packaging construct (Gag/Pol, predominantly) form the capsid and polymerase components and recognize specific cis-acting sequences in the retroviral RNA genome and its reverse-transcribed DNA products. This recognition leads to reverse transcription and integration. A separate construct expresses a heterologous envelope that is incorporated into the vector particles (pseudotyping) and allows entry into the target cells. A third construct, the transfer vector, contains retroviral cis-acting elements required for packaging by the vector particles, reverse transcription, nuclear translocation and integration in the target cells. It transfers an expression cassette for the gene(s) of interest containing an internal promoter (Fig. 1.10). The three expression constructs are maintained in the form of bacterial plasmids and can be transfected into mammalian cells to produce replication-defective virus stocks (Naldini et al., 1996).

Packaging construct

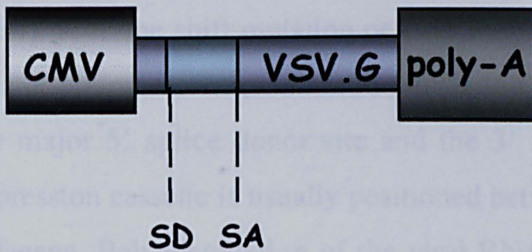
Early versions of packaging plasmids encoded most viral structural and regulatory proteins with the exception of the envelope glycoprotein (Naldini et al., 1996). A deletion within the 5' untranslated region upstream of *gag* and within the encapsidation signal was engineered to reduce the efficiency of incorporation of the helper RNA and thereby prevent recombination between vector and helper RNA molecules. The viral LTR promoter was replaced with a heterologous promoter such as the cytomegalovirus immediate early promoter. The 3' LTR was replaced with a heterologous polyadenylation signal (e.g., from SV40 or the insulin gene or another gene). A deletion

or mutation within Env ensured that no envelope glycoprotein was synthesized. The helper plasmid still expressed all other regulatory and accessory proteins of HIV-1. More recent variants of packaging plasmids have further deletions and modifications to eliminate all the accessory genes (Kim et al., 1998; Zufferey et al., 1997). The goal of creating these minimal constructs is to design helper constructs that express only those genes that are essential for gene transfer into a wide variety of cells at high efficiency. Early versions of the packaging plasmids contained the RRE and also expressed Rev. In order to further enhance safety, packaging systems have been devised in which the HIV-1 gag/pol and rev sequences have been segregated onto separate plasmids (Dull et al., 1998). Tat has also been eliminated from this system by using a Tat-independent gene transfer vector (see section 1.3.4). Such a packaging system uses four plasmids for the creation of vector stocks instead of three. This increases the safety of the system by further decreasing the probability of recombination between different helper plasmids and gene transfer vectors to recreate a replication-competent virus because multiple recombination steps need to occur for this to happen.

PACKAGING CONSTRUCT



ENVELOPE CONSTRUCT



TRANSFER VECTOR

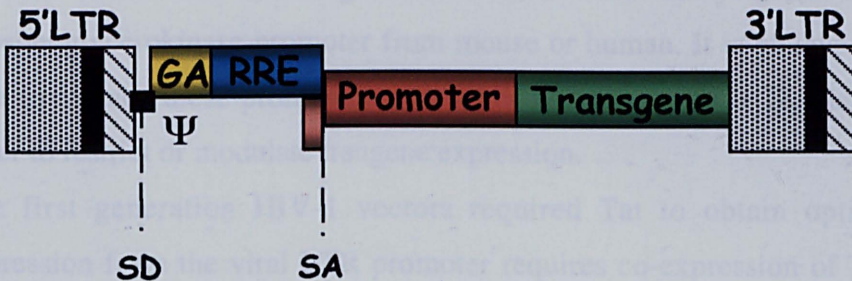


Figure 1.10. Elements of a prototypical Lentiviral Vector. The packaging construct expresses the gag and pol genes from the CMV promoter and intervening sequences and polyadenylation site of the human β -globin gene. As the transcripts of the gag and pol genes contain cis-repressive sequences, they are expressed only if Rev promotes their nuclear export by binding to the RRE. The envelope construct, encodes a heterologous envelope to pseudotype the vector, here shown coding for VSV-G. The transfer construct contains HIV-1 cis-acting sequences and an expression cassette for the transgene. It is the only portion transferred to the target cells. Only the relevant parts of the constructs are shown.

Gene Transfer Vector

The ideal gene transfer vector will lack all viral protein coding sequences and have a capacity for large transgenes. It should possess, in addition to the transgene expression cassette, all cis-acting sequences for efficient packaging, nucleocytoplasmic transport, reverse transcription and integration of the vector RNA. The first-generation vectors carried a deletion between the middle of the gag coding region and extending into the env coding region, thereby essentially eliminating all viral coding sequences (Naldini et al., 1996). It may not be possible to eliminate gag sequences completely because the sequence at the beginning of this gene has been shown to contain elements that can increase packaging efficiency of the vector RNA (Clever et al., 1995; Luban and Goff, 1994). A frame shift mutation or stop codon inserted within the gag reading frame can ensure premature termination of translation. The vectors also contain the RRE between the major 5' splice donor site and the 3' tat/rev splice acceptor site. The transgene expression cassette is usually positioned between the second coding exon of rev and the nef gene. Polyadenylation of the viral RNA (including that derived from the internal promoter) occurs using signals present in the 3' U3 and R sequences within the LTR. The most common internal promoter enhancer elements used in HIV-1 vectors are those derived from the cytomegalovirus immediate early gene and the cellular phosphoglycerokinase promoter from mouse or human. It is believed that one may be able to replace these promoters with other tissue-specific or regulatable elements, in order to restrict or modulate transgene expression.

The first generation HIV-1 vectors required Tat to obtain optimal titers, since expression from the viral LTR promoter requires co-expression of Tat. To overcome this, hybrid promoters that use enhancer elements from other viruses, instead of those present in the U3 of the HIV-1, have been created (Dull et al., 1998; Kim et al., 1998). These vectors appear to be nearly as efficient in terms of vector titer as the original Tat-dependent vectors that contained the wild type HIV-1 LTR. Although there is a report showing that Tat, in addition to its effect on transcription from the viral LTR, can also affect the efficiency of reverse transcription (Harrich et al., 1997) studies using HIV vectors have not revealed this requirement (Dull et al., 1998; Kim et al., 1998; Srinivasakumar and Schuening, 1999).

Figure 1.11 shows various modifications of gene transfer vectors to improve safety and expression. Considerable progress in biosafety was achieved by the successful generation of self-inactivating LVs. It has been shown that most of the U3 region of the

3' LTR can be safely deleted without compromising vector titer (Iwakuma et al., 1999; Zufferey et al., 1998). Such a deletion ensures that transcription from the 5' LTR promoters is efficiently suppressed following reverse transcription and integration into the target cell genome. Another advantage of using vectors with deletion of the U3 region is enhanced transgene expression from internal promoters (Zufferey et al., 1998). This is probably due to the decrease in promoter competition between the viral LTR and the internal promoter.

Further elements were also incorporated in the expression cassette to improve its performance (Fig. 1.11). Hepatitis B virus contains cis-acting elements that can substitute for Rev and RRE function in HIV-1 subgenomic constructs (Donello et al., 1996; Huang and Liang, 1993; Huang and Yen, 1994). Experiments have revealed that addition of a regulatory element from woodchuck hepatitis virus (WPRE) in retroviral and lentiviral vectors downstream of the transgene can increase expression by five to eight fold (Zufferey et al., 1999). This element acts at the post-transcriptional level, in all probability promoting the efficiency of polyadenylation of the nascent transcript, increasing the total amount of mRNA in the cells (Donello et al., 1998). Addition of the WPRE to HIV-derived vectors improved substantially the level of transgene expression from several types of promoter *in vitro* and *in vivo* (Deglon et al., 2000; Zufferey et al., 1999).

A recently described modification of the transfer vector backbone restored the infectivity of vector particles to the level of wild-type virus (Follenzi et al., 2000; Zennou et al., 2000). An additional copy of the polypurine tract, which primes transcription of the viral plus strand DNA, is present in the middle of the HIV genome, included in the pol coding region (Fig. 1.11). This sequence (cPPT) was shown to be required for efficient replication of HIV-1 by mutational analysis (Charneau et al., 1992; Ilyinskii and Desrosiers, 1998). Addition of this cPPT in the backbone of the transfer vector improved viral transduction in both dividing and nondividing cells.

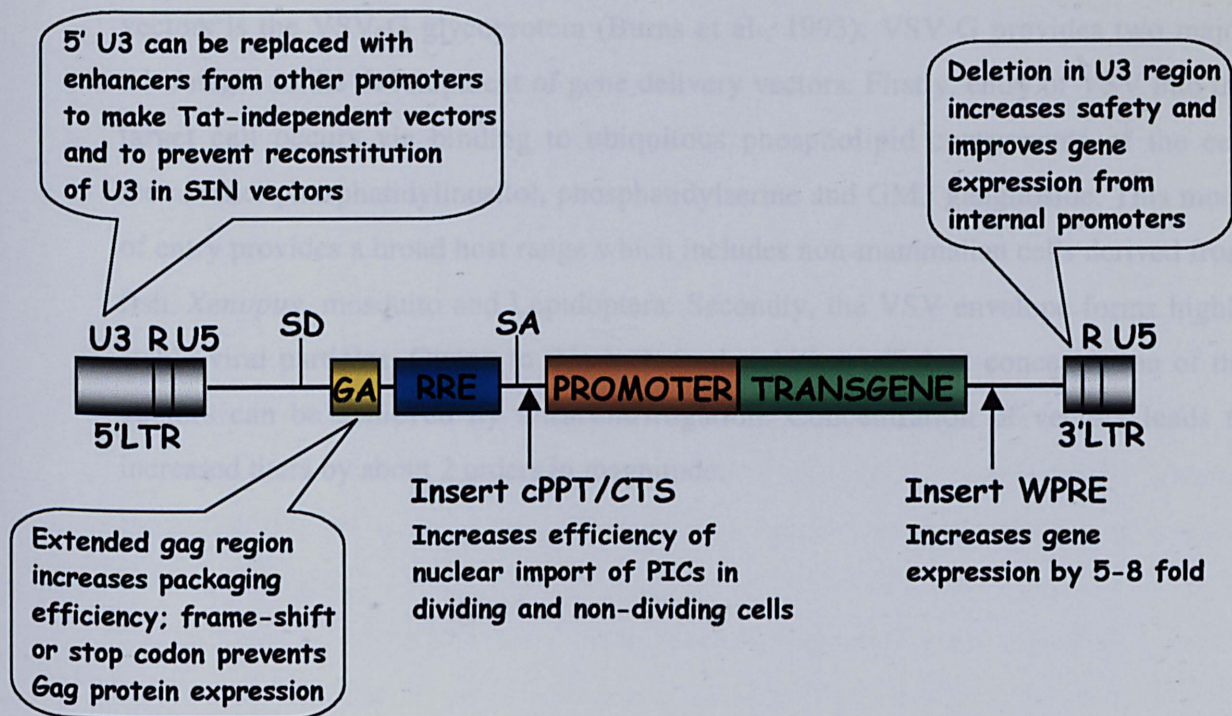


Figure 1.11. Schematic representation of a gene transfer vector based on HIV-1 and modifications to improve safety and expression (see text for details). LTR: long terminal repeat; SD: splice donor; SA: splice acceptor; SIN: self-inactivating; RRE: REV responsive element; WPRE: woodchuck post-transcriptional regulatory element; cPPT/CTS: central polypurine tract/central termination sequence.

Envelope construct

The most popular envelope glycoprotein that has been used for pseudotyping lentiviral vectors is the VSV-G glycoprotein (Burns et al., 1993). VSV-G provides two major advantages to the development of gene delivery vectors. Firstly, entry of VSV into the target cell occurs via binding to ubiquitous phospholipid components of the cell membrane: phosphatidylinositol, phosphatidylserine and GM3 ganglioside. This mode of entry provides a broad host range which includes non-mammalian cells derived from fish, *Xenopus*, mosquito and Lepidoptera. Secondly, the VSV envelope forms highly stable viral particles. Owing to this increased stability, efficient concentration of the vectors can be achieved by ultracentrifugation. Concentration of vectors leads to increased titers by about 2 orders in magnitude.

1.2.3.2 Safety characterisation

Because lentiviruses are potentially lethal human pathogens, the most urgent issue regarding the safety of LV vectors is their potential for recombination leading to replication-competent retrovirus (RCR), also referred to as "helper" virus. Generation of helper virus in preparations of replication-defective vectors has been documented in numerous instances involving oncoviruses (Donahue et al., 1992; Vanin et al., 1994). In later generations of vectors in which viral protein-coding regions were divided in the packaging cells, the frequency of recombination leading to helper was decreased, but not eliminated (Otto et al., 1994). Helper virus has the potential for inducing pathogenesis as demonstrated by studies in which monkeys were infused with transduced bone marrow cells after ablation of endogenous marrow with gamma irradiation (Purcell et al., 1996). In these studies, helper virus gave rise to lymphoma in monkeys.

Contemporary LV vectors were made virtually helper-free by segregating vector components into four plasmids (see section 1.2.3.2).

Another important safety issue involves the activation of cellular proto-oncogenes by the inserted provirus (Coffin et al., 1997). The genetic elements that can cause such insertional activation include the promoter, enhancer and the poly(A) site of the virus. For example, leukemia viruses (e.g. ALV) have been shown to induce lymphomas via the activation of c-myc expression by the promoter of the provirus (Fung et al., 1981). Theoretically, insertion of HIV-1 LTR's might lead to transcriptional activation of heterologous genes. However, this has not been yet demonstrated. To address the issue of insertional activation, self-inactivating (SIN) vectors were developed (Yu et al., 1986). The same principles were applied to recent LV vectors (Miyoshi et al., 1998; Zufferey et al., 1998). For example, self-inactivating vector described by Miyoshi et al. was constructed by deleting 133 bp in the U3 region of the 3' LTR including the TATA box and the binding sites for Sp1 and NF-kB. This deletion not only minimizes expression of vector RNA, but may also be useful in preventing the insertional activation of cellular proto-oncogenes by the integrated provirus.

1.3 Hemoglobinopathies

Hemoglobinopathies are a group of inherited disorders characterized by the absence of functional α -like or β -like globin chains (Stamatoyannopoulos, 2001). Sickle cell disease (SCD) and β -thalassemias are two of the most common categories of hematopoietic diseases. SCD include sickle cell anemia, sickle cell-hemoglobin C disease and sickle cell- β -thalassemia. Millions worldwide are affected; one of 400 African Americans, over 70,000 victims, are afflicted. These diseases are major health problems, associated with severe morbidity, lower-than-average life expectancy and serious, long-term disability. Clearly, it is of interest to combat these deadly diseases.

In the circulatory system, erythrocytes (red blood cells) transport oxygen to bodily tissues and carbon dioxide to the lungs for exhalation. Within erythrocytes, this process is mediated by hemoglobin, a molecule that consists of two α -like and two β -like globin chains and four iron-coordinated heme moieties. The human α -like and β -like globin loci, located on chromosomes 16 and 11 respectively, encode these protein chains. During development, different α - and β -globin genes are expressed to produce a developmental stage-specific hemoglobin molecule that meets the oxygen demand of the developing fetus (Fig. 1.12). Naturally occurring mutations within these loci cause the production of abnormal hemoglobins and anemia. Abnormal hemoglobins appear in one of three basic circumstances:

1. Structural defects in the hemoglobin molecule. Often, mutations in the gene for one of the two hemoglobin subunit chains, α or β , change a single amino acid building block in the subunit. Most commonly the change is innocuous, perturbing neither the structure nor function of the hemoglobin molecule. Occasionally, alteration of a single amino acid dramatically perturbs the behavior of the hemoglobin molecule and produces a disease state. Sickle hemoglobin exemplifies this phenomenon.

2. Diminished production of one of the two subunits of the hemoglobin molecule. Mutations that produce this condition are termed “thalassemias”. Equal numbers of hemoglobin α and β chains are necessary for normal function. Hemoglobin chain imbalance damages and destroys red cells thereby producing anemia. Although there is a dearth of the affected hemoglobin subunit, with most thalassemias the few subunits synthesized are structurally normal.

3. Abnormal associations of otherwise normal subunits. A single subunit of the α chain (from the α -globin locus) and a single subunit from the β -globin locus combine to produce a normal hemoglobin dimer. With severe α -thalassemia, the β -globin subunits

begin to associate into groups of four (tetramers) due to the paucity of α -chain partners. These tetramers of β -globin subunits are functionally inactive and do not transport oxygen. No comparable tetramers of α -globin subunits form with severe β -thalassemia. Alpha subunits are rapidly degraded in the absence of a partner from the β -globin gene cluster (γ , δ , β globin subunits).

1.3.1 The Thalassemias

The thalassemias are defined as a heterogeneous group of inherited disorders of hemoglobin synthesis, all characterized by the absence or reduced output of one or more of the globin chains of hemoglobin. They are most prevalent in the Mediterranean region, the Middle East, the Indian subcontinent and South-East Asia, representing a serious health problem in certain areas where gene frequencies reach 3-10% of the population (Weatherall and Clegg, 1996). They can be classified at several levels. First, there is a clinical classification, which simply describes the degree of severity. Second, the thalassemias can be defined by the particular globin chain that is synthesized at a reduced rate (genetic classification). Finally, it is now often possible to subclassify them according to the particular mutation that is responsible for defective globin chain synthesis (molecular classification).

Based on clinical features, the thalassemias are divided into the major forms of the illness, which are severe and transfusion-dependent and the symptomless minor forms, which usually represent the carrier state, or trait. Thalassemia major results either from the homozygous inheritance of a particular mutation or from the compound heterozygous state of two different mutations. Thalassemia intermedia describes conditions that are associated with a more severe degree of anemia than the trait, although they are not as severe as the major forms. Finally, there are some forms of thalassemia trait that are clinically and hematologically completely silent; they are designated as a silent carrier state.

According to their genetic basis the thalassemias are classified into α , β , γ , δ and $\epsilon\gamma\delta\beta$ varieties, depending on which chain or chains are synthesized at a reduced rate.

As their molecular pathology has been ascertained, it is now feasible to develop a more accurate approach to the designation of different types of thalassemias. For example, in many cases, it is possible to describe the genotype of a patient with the clinical picture of β -thalassemia major according to the particular mutations at the homologous pairs of β -globin chain loci.

1.3.1.1 β -Thalassemia

The β -thalassemias are the most intensively studied monogenic disorders in man. Over 180 different mutations that giving rise to the clinical phenotype of β -thalassemia have been identified (Olivieri, 1999). These mutations can be classified in two distinct categories: β^0 mutations, in which no β -globin chains are produced and β^+ mutations, in which some β chains are produced but at a reduced rate. The deficiency or absence of β -globin chains reflects the action of mutations that affect every level of β -globin gene function; that is, transcription, mRNA processing, translation and post-translational stability of the β -globin chain product. These mutations are classified according to the mechanism by which they affect β -globin gene expression.

The pathophysiology of β -thalassemia is directly linked to the degree of imbalance in the production of α and β -globin chains (Weatherall, 2001). In the most severe form of β -thalassemia found in homozygotes and compound heterozygotes, β -thalassemia major, the profound anemia requires regular lifelong blood transfusions; without treatment, the condition is lethal within the first year of life. In β -thalassemia intermedia, the anemia is less profound and may only require occasional transfusions. Chronic transfusions aimed to correct the anemia, suppress massive erythropoiesis and inhibit increased gastrointestinal absorption of iron. However, transfusion therapy leads to iron overload, which primarily affects the liver, heart and endocrine tissues. The iron overload is lethal if untreated and its prevention is the major goal of current patient management. At present, the only means to cure the disease definitively is through allogeneic bone marrow transplantation (Lucarelli et al., 2001).

1.4 The β -globin gene cluster

The human β -globin gene locus is the subject of intense study and over the past two decades a wealth of information has accumulated on how tissue-specific and stage-specific expression of its genes is achieved. The five genes of the human β -globin locus are arranged in a linear array on chromosome 11 and are expressed in a developmental stage-specific manner in erythroid cells (Fig. 1.12; Stamatoyannopoulos & Grosfeld, 2001).

The complex program of transcriptional regulation leading to the differentiation and developmental stage-specific expression in the globin locus is mediated by DNA-regulatory sequences located both proximal and distal to the gene-coding regions. The most prominent distal regulatory element in the human β -globin locus is the locus control region (LCR), located from about 6 to 22 kb upstream of the ϵ -globin gene (Forrester et al., 1987; Grosfeld et al., 1987). The LCR is composed of several domains that exhibit extremely high sensitivity to DNase I digestion in erythroid cells (called hypersensitive, or HS, sites), and is required for high-level globin gene expression at all developmental stages (Higgs, 1998).

1.4.1 Developmental Stage-specific expression of the β -globin genes

Over the last 40 years, an immense body of descriptive and experimental evidence has been reported in an effort to understand the biological phenomenon of hemoglobin switching. As reported more than 20 years ago by Nienhuis and Stamatoyannopoulos (Nienhuis and Stamatoyannopoulos, 1978), the phrase hemoglobin switching represents “the gradual replacement, in the blood stream, of red cells containing predominantly one hemoglobin with red cells which contain predominantly another”. Their definition articulates the now classic correlation among changes in red cells, their site of production and their globin content during human ontogeny. This switching paradigm is illustrated in Figure 1.12 B (Wood, 1976).

The five genes of the human β -globin gene locus are arranged in the same order as they are expressed during development (Fig. 1.12). The ϵ -globin gene is expressed in the embryonic yolk sac and located at the 5' end. After the switch in the site of hematopoiesis from the yolk sac to the fetal liver, the ϵ -gene is repressed and the two γ -globin genes, located downstream of ϵ , are activated. In a second switch, completed shortly after birth, the bone marrow becomes the major site of hematopoiesis, coincident with activation of the adult β -globin gene, while the γ -globin genes become

silenced. The δ -globin gene is also activated in erythroid cells derived from bone marrow hematopoiesis but is expressed at levels less than 5% of that of the β -globin gene. Studies utilizing transgenic mice produced with a variety of constructs containing portions or the entire human β -globin locus have provided insight into the *cis*-control of globin gene switching. Knock-out mice bearing null mutations in a number of genes have identified several trans-acting proteins necessary for globin expression. The individual genes of the human β -globin locus are developmentally regulated through their proximal promoters, but expression in transgenic mice is usually low and variable due to position of integration effects. Position effects are overcome by linkage to the LCR and high-level, position-independent expression is observed for the human γ - or β -globin genes (Enver et al., 1990; Grosveld et al., 1987). Genetic information governing the stage-specificity for all β -like globin genes is located in gene proximal regions. These elements represent transcription factor-binding sites that recruit proteins or protein complexes in a stage-specific manner. Examples exist for the presence of both positive and negative acting factors that turn genes on or off at a specific developmental stage.

1.4.2 The β -globin locus control region

The β -globin LCR (locus control region) is composed of five HS sites located upstream of the ϵ gene within a 20 kb region (Fig. 1.12). The presence of HS indicates that *trans*-acting factors are binding to these regions and displacing or destabilizing nucleosomes. Expressed genes are located in an “open” chromatin context more accessible to *trans*-acting factors and in general containing nucleosomes with highly acetylated histones. In contrast, “closed” chromatin generally has deacetylated histones and is bound by the linker histone H1, is less accessible to DNA binding factors and genes in these regions are not expressed. Mechanisms by which chromatin structure controls activation and repression of transcription are not well understood. Proteins that bind and activate transcription may access DNA without disruption of the nucleosome or they may require the assistance of protein cofactors that function to make DNA more accessible by modifying the nucleosome (Kingston and Narlikar, 1999; Tyler and Kadonaga, 1999). Other less defined parameters such as the nature of the inherent DNA sequence and the stage of the cell cycle, influence the structure of chromatin (Peterson and Logie, 2000).

The mechanism by which the LCR controls β -globin gene expression has been extensively studied, primarily using transgenic and Knock-out mice (Stamatoyannopoulos & Grosveld, 2001). It is important to realize that these two assays manipulate the genes in quite different ways and the results are not always complementary or in agreement with each other. Transgenic mice contain the human β -globin gene transferred into novel or ectopic integration sites; whereas, Knock-out mice manipulate the endogenous native locus in the mouse. In transgenic mice, human β -globin transgenes are silent at most integration sites or transcribed to about 1% of the endogenous mouse β -major level. In contrast, addition of the LCR including all four erythroid specific HSs to the β -globin transgene results in expression to about 100% levels at all integration sites, and expression is copy number-dependent (Ellis et al., 1996; Grosveld et al., 1987; Talbot et al., 1989). This copy number-dependent, position-independent transgene expression is the defining feature of LCR activity. Further investigation demonstrated that individual HS2, HS3 and HS4 elements and their smaller “cores” of approximately 200-300 bp, also direct copy number-dependent transgene expression but to lower levels (10-25%) (Philipsen et al., 1993; Talbot and Grosveld, 1991).

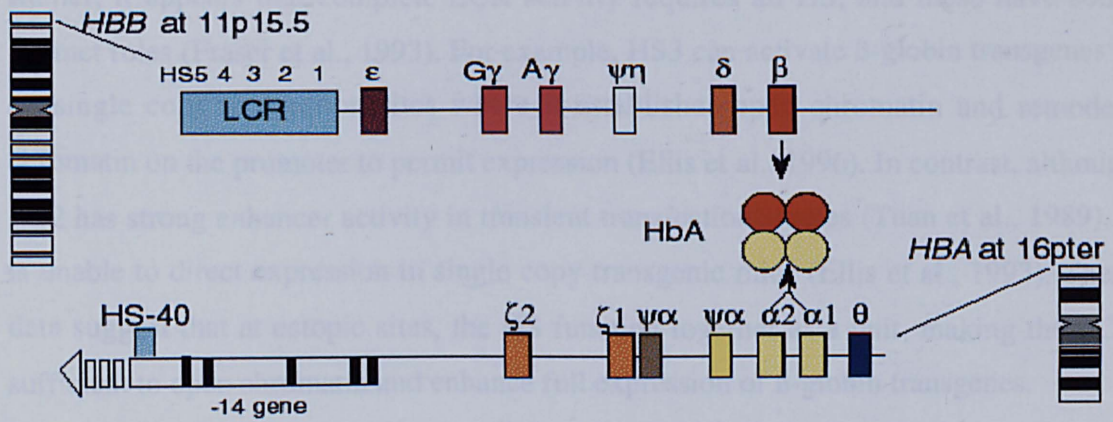
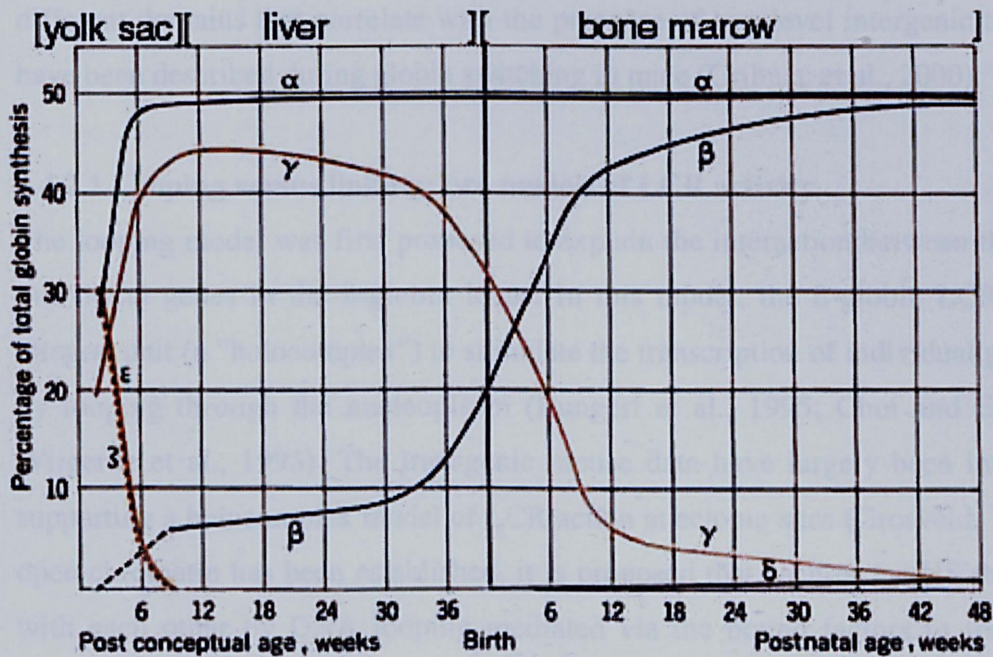
A**B**

Figure 1.12. (A) Genomic organization of human globin genes. (B) Haemoglobin synthesis and sites of haematopoiesis during human fetal development.

The LCR is often referred to as an enhancer, but does not have classic enhancer activity because it does not function equally well in either orientation (Tanimoto et al., 1999). Rather, it appears that complete LCR activity requires all HS, and these have some distinct roles (Fraser et al., 1993). For example, HS3 can activate β -globin transgenes at all single copy integration sites where it establishes open chromatin and remodels chromatin on the promoter to permit expression (Ellis et al., 1996). In contrast, although HS2 has strong enhancer activity in transient transfection studies (Tuan et al., 1989), it is unable to direct expression in single copy transgenic mice (Ellis et al., 1993). These data suggest that at ectopic sites, the HS function together as a unit, making the LCR sufficient to open chromatin and enhance full expression of β -globin transgenes. Open chromatin is likely to be established by the binding of erythroid *trans*-acting factors that recruit chromatin remodelling complexes (Blobel et al., 1998; Armstrong et al., 1998; Gong et al., 1996), as has been described for histone acetylation changes on active human β -globin genes (Schubeler et al., 2000). This open chromatin may not extend throughout transgenes containing the entire human LCR β -globin cluster, as different domains that correlate with the presence of low level intergenic transcription have been described during globin switching in mice (Gribnau et al., 2000).

1.4.2.1 Looping versus linking: two models of LCR activity

The looping model was first proposed to explain the interaction between the LCR and individual genes in the β -globin locus. In this model, the β -globin LCR acts as an integral unit (a “holocomplex”) to stimulate the transcription of individual globin genes by looping through the nucleoplasm (Bungert et al., 1995; Choi and Engel, 1988; Wijgerde et al., 1995). The transgenic mouse data have largely been interpreted as supporting a holocomplex model of LCR action at ectopic sites (Grosveld, 1999). Once open chromatin has been established, it is proposed that each of the HS then interacts with each other by DNA looping mediated via the bound factors to form an LCR holocomplex (Fig. 1.13 A). The holocomplex would then interact with a single globin gene in the cluster, and switching during development would be accomplished by stage-specific silencer elements associated with the e- and g-globin gene promoters. A key question in the looping model is whether there is a direct interaction between LCR and promoters. Previous support for this model has come from several types of studies. The ability of an enhancer to activate a promoter located on a physically separate DNA molecule, is most easily explained by direct contact between the enhancer and the gene.

This has been observed in transvection in *Drosophila* (Bickel and Pirrotta, 1990) and in a number of *in vitro* experiments with artificial DNA constructs (Dunaway and Droge, 1989; Mahmoudi et al., 2002). Competition between genes for a single regulator (Hanscombe et al., 1991; Wasylyk et al., 1983), leading to alternate transcription is also most easily explained by looping, particularly because the competitive advantage of the enhancer-proximal gene is lost when the genes are closely spaced at distances further from the enhancer (Heuchel et al., 1989). However, all this evidence is indirect, and each can also be explained by other mechanisms. Nevertheless, very recent data from Grosveld lab demonstrated that the distal regulatory elements and the active genes, which are linked *in cis* in the murine β -globin locus, interact *in vivo* while the intervening DNA loops out. This looping is only seen in expressing cells and provides direct *in vivo* evidence for the looping model (Tolhuis et al., 2002). In particular, the observation that two hypersensitive sites at the far ends of the region cluster with the LCR and the active genes (i.e., all hypersensitive sites) provides new insights into long-range interactions. On the basis of these data they propose that direct interactions between distal DNase I hypersensitive sites and looping out of chromatin is crucial in establishing an open chromatin domain and activating transcription. Furthermore, evidences suggesting long-range chromatin regulatory interactions has been provide *in vivo*. In fact, Fraser lab shows that the HS2 enhancer element of the β -globin LCR is in close physical proximity to an actively transcribed β -globin gene located over 50 kb away (Carter et al., 2002).

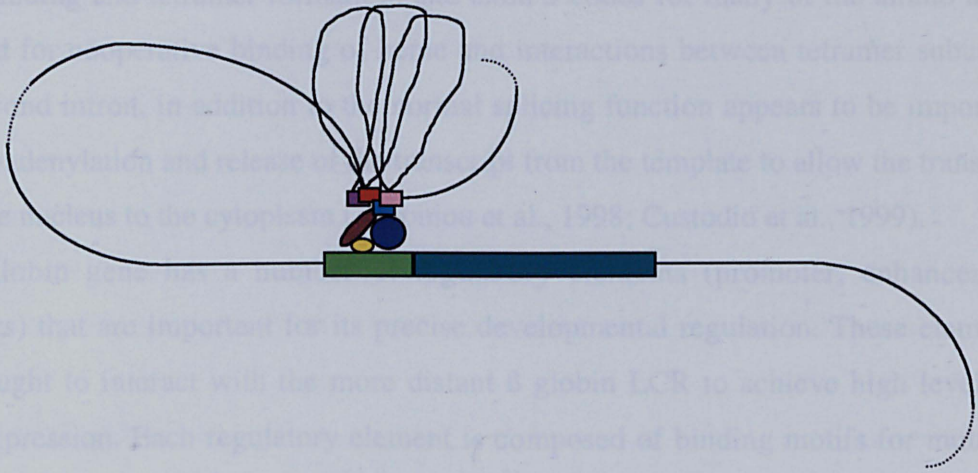
Quite different conclusions have been arrived at using knock-out technology on the mouse β -globin locus (Higgs, 1998). Deletions of individual or all the HS in the endogenous locus do not alter chromatin structure and have relatively minor effects on expression of the globin genes (Epner et al., 1998; Reik et al., 1998). These data suggest that the LCR is not required for chromatin opening at the endogenous mouse β -globin locus and suggest that more distant elements control chromatin structure (Bulger and Groudine, 1999). A linking model that does not invoke DNA looping has been proposed to explain the knockout results (Fig. 13 B). In this model, the function of the LCR is to enhance β -globin expression by ensuring that factors are bound at intervals across the cluster and that the gene is localized to the right nuclear compartment. The linking model is not consistent with the ability of the LCR to open chromatin at ectopic transgene sites, but the holocomplex model cannot easily explain the effect of LCR

deletions in the mouse β -globin locus. However, the two models are not necessarily mutually exclusive and may be strengthened by being merged.

Several recent chromatin immunoprecipitation (ChIP) analyses of factor binding in the β -globin locus of cultured erythroid cell lines have provided new clues to understanding the regulation of β -globin gene expression (Bulger et al., 2002; Johnson et al., 2001). It was suggested that hyperacetylation at the β -globin gene promoter is partially NF-E2 dependent and the association of RNA polymerase II is wholly NF-E2 dependent. The hyperacetylation of LCR is NF-E2 independent and RNA polymerase II is associated with the β -globin LCR prior to NF-E2 binding. Based on this data, a three-step model for β -globin gene activation was proposed (Johnson et al., 2001; Sawado et al., 2003).

For the purpose of gene therapy where globin expression cassettes delivered by viral vectors must express at ectopic sites, it will be important to design the cassettes based on transgene constructs that express to high levels as single copy integration events.

A) Looping



B) Linking

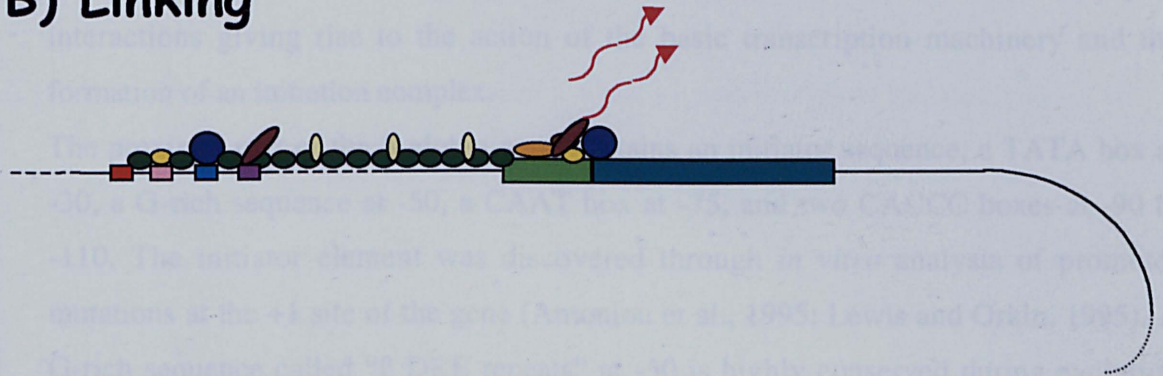


Figure 1.13. Proposed models for LCR-globin gene interaction. A globin gene is denoted as a green rectangular box with the promoter region indicated in a lighter green. Transcription factors are shown as colored ovals and circles. The four erythroid-specific hypersensitive site cores (HSs) are indicated by small colored boxes. The flanking DNA sequences of the HSs are depicted as loops between the HS cores. Transcripts are denoted by wavy arrows. (A) Looping model. Transcription factors bind to the LCR HSs and the gene promoter. The LCR directly interacts with the gene promoter by looping out the intervening DNA, thus forming an active transcription complex at the gene promoter. (B) Linking model. Sequential binding of transcription factors along the DNA directs changes in chromatin conformation and defines the transcriptional domain. The transcription factors are linked to one another from the LCR to the gene promoter by non-DNA-binding proteins and chromatin modifiers (shown as colored ovals and circles).

Adapted from Harju S *et al.*, 2002.

1.4.3 Regulation of the β -globin genes

The β -globin gene is a relatively small gene comprising three coding exons and two introns. The exons code for 146 amino acids and correspond to functional domains in the protein. For example, exon 2 codes for the part of the globin chains involved in heme binding and tetramer formation, and exon 3 codes for many of the amino acids required for cooperative binding of heme and interactions between tetramer subunits. The second intron, in addition to the normal splicing function appears to be important for polyadenylation and release of the transcript from the template to allow the transport from the nucleus to the cytoplasm (Antoniou et al., 1998; Custodio et al., 1999).

Each globin gene has a number of regulatory elements (promoter, enhancer, or silencers) that are important for its precise developmental regulation. These elements are thought to interact with the more distant β globin LCR to achieve high levels of gene expression. Each regulatory element is composed of binding motifs for multiple erythroid restricted and ubiquitously expressed transcriptional activators or suppressor. These factors interact and synergize with each other and other cofactors resulting in the formation of multimeric complexes that change chromatin structure to allow complex interactions giving rise to the action of the basic transcription machinery and the formation of an initiation complex.

The proximal part of the β -globin gene contains an initiator sequence, a TATA box at -30, a G-rich sequence at -50, a CAAT box at -75, and two CACCC boxes at -90 to -110. The initiator element was discovered through *in vitro* analysis of promoter mutations at the +1 site of the gene (Antoniou et al., 1995; Lewis and Orkin, 1995). A G-rich sequence called " β DRE repeats" at -50 is highly conserved during evolution (Stuve and Myers, 1990). Mutations of the repeats affect promoter function in transfection assays in erythroid cells and a DNA binding activity has been associated with the element (Myers et al., 1989).

The CAAT box region is important for promoter function in erythroid cells (Antoniou and Grosveld, 1990), and has been shown to bind several different factors: the erythroid-specific protein GATA-1, the ubiquitous CAAT-binding protein CP1 (also called NF-Y) and a DNA binding activity that was denoted DSFr (Delvoye et al., 1993). CP1 is thought to be a positive regulator of the CAAT box, but this has not been confirmed *in vivo*. GATA-1 binds fairly weakly at the CAAT box and probably is not functionally important at this position. DSFr is involved in the up-regulation of

transcription and in MEL cells was identified as C/EBP-g through expression cloning and antibody studies (Wall et al., 1996).

Of the two CACCC boxes, the proximal appears to be more important for their function. The CACCC box binds several factors *in vitro* (Hartzog and Myers, 1993), but the functional protein *in vivo* is the transcription factor EKLF. The β -globin CACCC box has a higher binding affinity for EKLF than the ϵ or γ -globin CACCC boxes and mutations that substantially decrease the binding affinity of the CACCC box for EKLF produce a thalassemic phenotype (Donze et al., 1995).

The upstream part of the promoter contains additional sites for GATA-1 (at -120 and -200) and CP1 (at -160), and these appear to be important for inducible β -globin promoter activity in erythroleukemia cells (Antoniou and Grosveld, 1990; deBoer et al., 1988).

The β -globin gene has been reported to contain two enhancer elements, one located near the junction of the second intron and the third exon and another a 6 hundred base pairs downstream from the poly-A site of the gene (Antoniou et al., 1988; Behringer et al., 1987; deBoer et al., 1988; Wall et al., 1996).

1.4.3.1 Transcriptional regulation of β -globin genes during erythropoiesis

The tissue- and developmental-specific expression pattern of the individual globin genes is achieved through the action of transcription factors on regulatory sequences that are immediately flank the individual genes and on more distant sequences that are important for the regulation of all the genes of the locus. The factors that regulate globin genes are either tissue restricted or ubiquitous with respect to their expression pattern. Only a few factors have been studied in detail for their direct role in the transcription of the globin genes, many of them affect globin gene transcription through their importance in hematopoietic or erythroid differentiation in general (see Section 1.1.3.2). On the other hand, several factors important for globin gene transcription are ubiquitous and influence the expression of many genes, most of which are not erythroid specific.

GATA-1

GATA-1, a zinc finger transcription factor, plays a central role in erythroid development. It was first identified by its ability to bind functionally important DNA regulatory sequences found in globin genes (deBoer et al., 1988; Evans and Felsenfeld, 1989). Since then, GATA-binding motifs ((T/A)GATA(A/G)) have been identified in

the promoters and/or enhancers of virtually all erythroid and megakaryocytic-specific genes studied (Orkin, 1992; Weiss and Orkin, 1995). GATA-1 contains two zinc fingers, both of the Cys-X2-Cys-X17-Cys-X2-Cys configuration.

Expression of GATA-1 is restricted to erythroid, megakaryocytic, eosinophilic, mast and multipotential precursor cells within the hematopoietic system (Evans and Felsenfeld, 1989; Tsai et al., 1989). The sole non-hematopoietic site of expression is the Sertoli cells of the testis (Ito et al., 1993). Gene targeting studies in mice have shown that GATA-1 is essential for normal erythropoiesis (Pevny et al., 1991). GATA-1 hemizygous male knock-out mice (GATA-1 is located on the X-chromosome) die at mid-embryonic gestation (E10.5) from severe anemia with arrest in erythroid maturation at a proerythroblast-like stage (Fujiwara et al., 1996). Differentiated *in vitro* GATA-1⁻ ES cells likewise fail to mature past the proerythroblast stage and undergo rapid apoptosis, indicating a role for GATA-1 in cell survival as well as maturation (Weiss and Orkin, 1995). Loss of GATA-1 expression in megakaryocytes also leads to defects in maturation characterized by impaired endoreduplication and granule formation, disorganized platelet demarcation membrane synthesis and hyperproliferative growth (Shivdasani et al., 1997). In primitive erythroid cells, GATA-1 expression is regulated by a 5' enhancer, whereas its expression in definitive erythroid cells requires an additional element located in the first intron. Together, these two elements form the GATA-1 locus hematopoietic regulatory domain (HRD) (Shimizu et al., 2001).

The C-terminal and the N-terminal zinc-fingers (CF and NF, respectively) in GATA-1 are required for recognition of the GATA motif and DNA binding as well as for physical interaction with other transcription factors. The highly conserved NF is essential for interaction with the GATA-1 coactivator FOG (Friend Of GATA) as well as with EKLF, LMO2 and CREB binding protein (CBP), and enhances the specificity and stability of binding of the two-finger DNA binding domains to palindromic GATA recognition sequences (Fig.1.14) (Blobel et al., 1998; Crispino et al., 1999). CF is necessary for GATA-1 function, while NF is required for definitive but not for primitive erythropoiesis. This suggests that different GATA-1 functional domains are required for target gene activation in primitive and definitive erythropoiesis (Shimizu et al., 2001). Thus, both transcriptional regulatory elements and protein functional domains may ensure proper lineage specification in primitive and definitive erythropoiesis.

Binding of erythroid-specific transcription factors, such as GATA-1, to enhancers of erythroid-specific genes early in development or differentiation could be a key factor in initiation and maintenance of active chromatin structures (Martin et al., 1996). GATA-1 orchestrates multiple programs of erythroid development, including control of the cell cycle and apoptosis, as well as differentiation (see Section 1.4.3.1). At the biochemical level, GATA-1 is thought to recruit critical proteins to target sites. The interaction of GATA-1 with CBP/p300 suggests at least one mechanism by which histone acetylases might be brought to specific site (Blobel, 2000; Blobel et al., 1998). By modifying chromatin-bound histones or perhaps by modification of GATA-1 itself, acetylases could enhance transcriptional activity of erythroid-specific loci (Boyes et al., 1998).

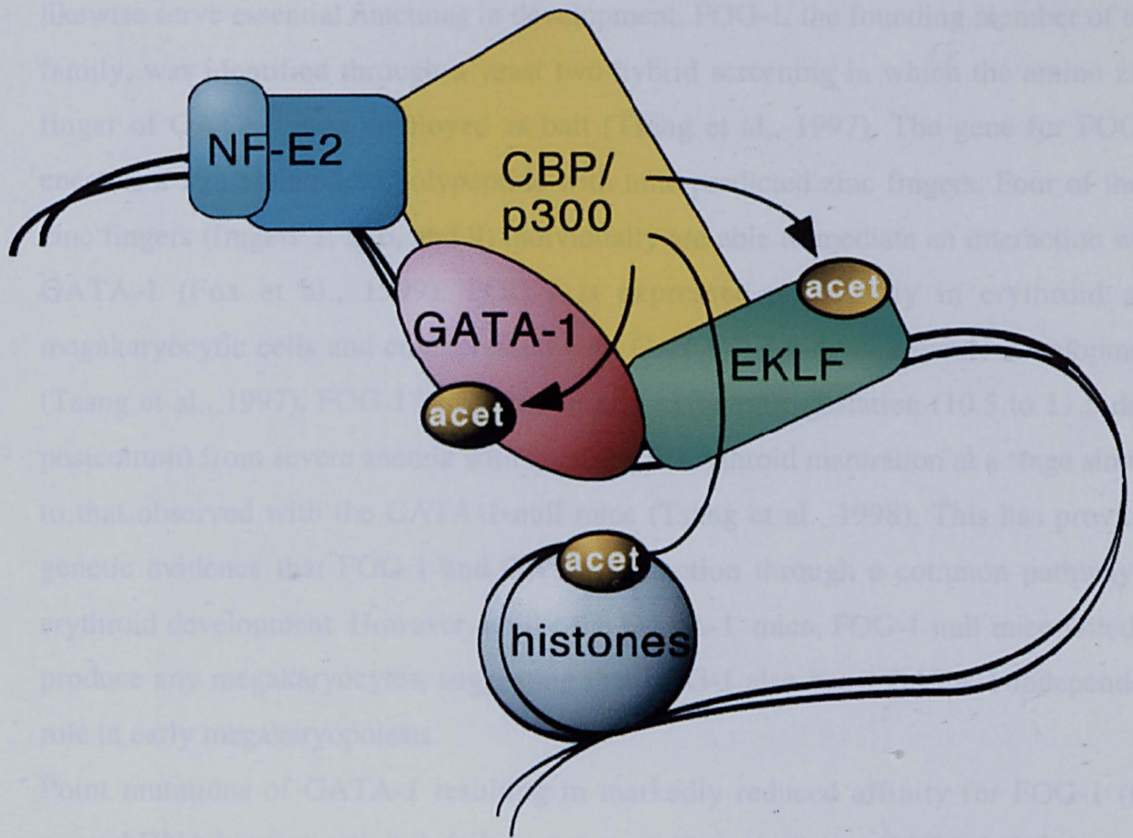


Figure 1.14. Hypothetical model of NF-E2, GATA-1 and EKLF cooperative interaction to recruit CBP and p300 to the β -globin LCR. This could lead to acetylation of nearby histones and transcription factors. Acetylation of histones leads to changes in chromatin structure, and acetylation of transcription factors might stabilize their interaction with DNA or alter their transcriptional activity. It is conceivable that this high molecular weight complex also connects to the promoters of the globin genes through a looping mechanism (from Blobel G, Blood, 2000).

Friend of GATA (FOG)

The FOG (Friend of GATA) family of proteins comprises a novel class of multi-type zinc finger nuclear polypeptides that interact physically with GATA factors and likewise serve essential functions in development. FOG-1, the founding member of this family, was identified through a yeast two-hybrid screening in which the amino zinc finger of GATA-1 was employed as bait (Tsang et al., 1997). The gene for FOG-1 encodes a 998-amino-acid polypeptide with nine predicted zinc fingers. Four of these zinc fingers (fingers 1, 5, 6, and 9) individually are able to mediate an interaction with GATA-1 (Fox et al., 1999). FOG-1 is expressed abundantly in erythroid and megakaryocytic cells and co-expressed with GATA-1 during embryonic development (Tsang et al., 1997). FOG-1^{-/-} mice die in mid-embryonic gestation (10.5 to 11.5 days postcoitum) from severe anemia with an arrest in erythroid maturation at a stage similar to that observed with the GATA-1-null mice (Tsang et al., 1998). This has provided genetic evidence that FOG-1 and GATA-1 function through a common pathway in erythroid development. However, unlike the GATA-1^{-/-} mice, FOG-1 null mice failed to produce any megakaryocytes, suggesting that FOG-1 also has a GATA-1-independent role in early megakaryopoiesis.

Point mutations of GATA-1 resulting in markedly reduced affinity for FOG-1 (but normal DNA binding activity), fails to rescue erythropoiesis in a GATA-1-deficient cell line (Crispino et al., 1999). A similar point mutation in humans leads to severe congenital dyserythropoietic anemia and thrombocytopenia (Nichols et al., 2000). Such patients have an overabundance of abnormal megakaryocytes resembling GATA-1-megakaryocytes. Taken together, these findings demonstrate that a direct physical interaction between GATA-1 and FOG-1 is critical for normal erythropoiesis as well as late stages of megakaryopoiesis.

A second mammalian member of the FOG family (FOG-2) is expressed predominantly in heart, brain, lung, and gonadal tissues (Tevosian et al., 1999). FOG-2^{-/-} mice die during embryogenesis from cardiac defects characterized by thin ventricular myocardium, Tetralogy of Fallot malformation, common atrioventricular valve and defective coronary vasculature (Tevosian et al., 2000). All GATA family members are capable of interacting with either FOG-1 or FOG-2 through conserved residues in their amino zinc fingers. Knock-in of a FOG non-interacting point mutation into the GATA-4 gene, one of the cardiac expressed GATA factors, results in embryonic death and a constellation of heart defects similar to that observed in the FOG-2^{-/-} mice (Crispino et

al., 2001). This indicates that interactions between GATA and FOG proteins are critical for multiple developmental processes.

Efforts have now been focused on understanding the mechanism by which FOG proteins influence GATA-mediated processes. Virtually all erythrocyte- and megakaryocyte-expressed genes contain GATA DNA-binding sites in both their promoters and enhancer elements (Orkin, 1992). Since a single FOG-1 molecule can potentially bind more than one GATA-1 molecule, FOG-1 might function as a molecular bridge between distant GATA-1-DNA complexes (Fox et al., 1999). This could bring distal enhancer elements into proximity of the promoter through DNA looping, a mechanism invoked in models of enhancer-promoter interactions and also in the activity of the β -globin LCR (Bulger and Groudine, 1999). However, mutants of FOG-1 that are capable of binding only a single molecule of GATA-1 rescue erythroid maturation from a FOG-1 deficient cell line as well as the wildtype molecule, providing evidence against such a model in its simplest form (Cantor et al., 2002). Another possibility is that FOG provides a transcriptional activation domain either by itself or via interaction with another transcriptional coactivator. This predicts that protein domains outside of the GATA-binding zinc fingers would be required for its activity. However, FOG-1 molecules containing extensive and overlapping deletions spanning the entire molecule (but retaining at least one GATA-binding zinc finger) are also able to rescue erythroid maturation of a FOG-1^{-/-} cell line (Cantor et al., 2002). This suggests that a simple interaction between FOG-1 and GATA-1 is by itself sufficient to activate GATA-1. This could occur through an allosteric change in GATA-1 or perhaps by the displacement of a repressor protein bound to GATA-1. Indirect support for the latter model is suggested by the earlier work of Evans and Felsenfeld, who reported that GATA-1 has reduced transcriptional activity in hematopoietic cells compared to non-hematopoietic cells, suggesting that the hematopoietic environment somehow dampens GATA-mediated response (Evans and Felsenfeld, 1991).

Nuclear Factor-erythroid 2 (NF-E2)

Nuclear Factor-erythroid 2 (NF-E2) was the second erythroid specific protein that was identified through DNA-binding studies *in vitro*. It was discovered through its binding to an AP-1 motif in the promoter of the porphobilinogen deaminase gene (Mignotte et al., 1989), and was somewhat ignored until it was shown that this factor could bind to the HS2 region of the β -globin LCR (Ney et al., 1990). NF-E2 is expressed in several

hematopoietic lineages, including erythroid, mast, and megakaryocytic cells. It is also expressed in the intestine of anemic mice and may be involved in iron metabolism (Andrews et al., 1993). NF-E2 is a 45 kDa protein with a basic DNA-binding domain, an adjacent leucine zipper domain, an N-terminal proline-rich domain, and a cap'n collar (CNC) domain necessary for transcriptional activation (Bean and Ney, 1997).

NF-E2 heterodimerizes with members of the family of small, ubiquitously expressed 18 kDa Maf proteins, which themselves have no transcriptional activation domain but which are essential for binding site recognition (Motohashi et al., 1997).

The overall stimulatory activity of the LCR (at least of HS2) in the chromatin environment of transgenic mice or in stably integrated constructs in cells appears to depend on NF-E2 motifs (Caterina et al., 1994; Caterina et al., 1994; Ellis et al., 1993; Talbot and Grosveld, 1991). A series of studies using transgenic mice with a β -globin yeast artificial chromosome (YAC), suggested that the deletion of HS elements markedly reduced the expression of all of the globin genes at all developmental stages accompanying the malformation of DNase I hypersensitivity in the LCR (Li et al., 1998; Milot et al., 1996). In addition, it was also shown that the protein Bach1, which heterodimerizes with the p18 NF-E2 subunit and interacts with MARE (Maf responsive element or NF-E2 binding site) sequences at HS2, HS3 and HS4, is able to cross-link HS sites, thereby looping out intervening DNA regions (Yoshida et al., 1999). These results suggest that a series of protein-protein and protein-DNA interactions establish the formation of a larger LCR complex (Wijgerde et al., 1995). Furthermore, NF-E2 is critically involved in remodeling the nucleosome structure over the HS2 region, where it interacts with the tandem MARE sites (Armstrong and Emerson, 1996; Gong et al., 1996). In contrast with the study supporting a role for NF-E2 in β -globin gene expression, p45 NF-E2-null mice had nearly normal levels of β -globin protein, and deletion of HS2 had no significant effect on the timing or extent of expression of the gene (Fiering et al., 1995). Disruption of one of the genes coding for one of the Maf subunits shows no phenotype, most likely because of compensation by one of the other Maf proteins (Kotkow and Orkin, 1996).

Erythroid Kruppel-like factor (EKLF)

Tissue- and stage-specific expression of the various globin genes is determined by the interactions between the LCR and the specific globin gene promoters, interactions mediated by recruiting chromatin modifying, coactivators and transcription complexes

(Levings and Bungert, 2002). The most extensively studied stage-specific activator is EKLF, which is crucial for human β -globin gene expression. The EKLF-DNA binding site consensus sequence 5'-NCNCNCCCN-3' corresponds to a functionally important motif within the adult β -globin gene promoter. Mutation of this sequence in humans is found in some patients with β -thalassemia. Targeted disruption of the EKLF gene in mice also results in lethal β -thalassemia. EKLF knock-out mice die from severe anemia at the fetal stage due to failure of adult β -globin gene activation. EKLF^{-/-} mice containing a complete human β -globin locus transgene have reduced levels of β -globin, but elevated levels of γ -globin expression, compared to wildtype mice containing the same transgene (Perkins *et al.*, 1996; Wijgerde *et al.*, 1996). Taken together, these experiments suggest that EKLF participates in the switch from embryonic/fetal globin to adult β -globin expression in humans.

New insight into EKLF function has come from experiments showing that EKLF requires the presence of a SWI/SNF-related chromatin remodeling complex (E-RC1, EKLF coactivator remodeling complex-1) for its tissue-specific regulation, indicating that it may affect transcription by altering chromatin configuration (Armstrong *et al.*, 1998). Reintroducing EKLF into an EKLF-null erythroid cell line, which harbors a copy of the human β -globin locus, resulted in enhanced differentiation and hemoglobinization, as well as reduced proliferation. This may point to a role for EKLF in cell cycle regulation and hemoglobinization, in addition to regulation of β -globin gene expression (Coghill *et al.*, 2001).

The Fli-1 oncogene

A member of the Ets family of transcription factors, Fli-1, was identified in Friend virus-induced erythroleukemia and affects the self-renewal of erythroid progenitor cells (Howard *et al.*, 1993). In pluripotent human hematopoietic cells, differentiation is followed by reduced Fli-1 expression and over expressing Fli-1 inhibits erythroid differentiation, impairs the cells' ability to respond to specific erythroid inducers such as hemin, and reduces the levels of GATA-1 (Athanasίου *et al.*, 2000). In the erythroblastic cell line HB60, Fli-1 expression is downregulated by erythropoietin (Epo), which induces terminal erythroid differentiation. Constitutive expression of Fli-1 blocks Epo-induced differentiation and enhances cell proliferation in HB60 cells, suggesting that Fli-1 targets erythroid cells to either proliferation or differentiation, in response to Epo (Tamir *et al.*, 1999). Fli-1 binds a cryptic Ets consensus site within the

retinoblastoma (Rb) gene promoter, repressing Rb expression, which results in impaired terminal erythroid maturation and continuous presence of nucleated erythrocytes in peripheral blood (Lee et al., 1992). Negative regulation of Rb by Fli-1 could destine erythroid progenitors to self-renewal, while Epo-induced repression of Fli-1 expression will enable differentiation (Tamir et al., 1999).

PU.1

The putative oncogene Spi-1 (PU.1) protein product is a hematopoietic-specific Ets factor, promoting differentiation of lymphoid and myeloid lineages (Scott et al., 1994). PU.1 expression in erythroid progenitors can induce erythroleukemia in mice. Like Fli-1, PU.1 blocks erythroid differentiation and restoration of terminal erythroid differentiation in murine erythroleukemia (MEL) cells requires PU.1 suppression (Rekhtman et al., 1999).

PU.1 can interact directly with GATA-1 and repress GATA-1 mediated transcriptional activation (see Section 1.1.3.2.3). Both the PU.1 DNA binding domain and transactivation domain are required for GATA-1 suppression and for blocking terminal differentiation in MEL cells. PU.1 does not seem to affect binding of other factors, such as FOG, to GATA-1, nor does it prevent GATA-1 DNA binding (Rekhtman et al., 1999). It is likely that PU.1 binds to assembled, DNA-bound GATA-1 complexes and represses their activity. Ectopic expression of PU.1 in *Xenopus* embryos blocks erythropoiesis. Exogenous GATA-1 is able to relieve this blockage of erythroid differentiation in MEL cells as well as in *Xenopus* embryos and explants, suggesting that lineage commitment decisions are regulated by their relative levels.

1.4.3.2 Putting the puzzle together: a model for β -globin gene regulation

A four-step model for human β -globin gene regulation has been suggested (Levings and Bungert, 2002) the first step involves partial unfolding of globin chromatin structure and generation of a highly accessible LCR. It is mediated by erythroid-specific proteins, which bind to sequences throughout the globin locus. GATA-1, which is known to associate with histone acetyl-transferases, may be involved in this step. The disruption of the LCR chromatin structure allows binding of transcription factors such as EKLF, GATA family members and the HLH proteins to the LCR HS sites, and the recruitment of chromatin-remodeling complexes and coactivators. In the third step, chromatin domains permissive for transcription are being established. Intergenic transcription was suggested to modify chromatin structure of an active gene domain, distinguishing it from an accessible but inactive one, that way separating the globin genes into developmental stage-specific chromatin domains. Finally, transcription complexes are being transferred from the LCR to individual globin gene promoters within transcriptionally permissive domains, allowing the developmental stage-specific pattern of globin gene expression.

1.5 Specific Aims

For most clinical applications, restricting the expression of a retrovirally delivered therapeutic transgene to a specific subset of cells within a tissue, or to specific progeny of a multipotent stem cell, is a mandatory requirement. This need can be achieved either by targeting vector entry into a specific cell subset (transductional targeting) and/or by targeting the expression of the transgene by appropriately regulating its transcription (transcriptional targeting). Redirecting retroviral particle entry can be achieved by either pseudotyping, genetically modifying the envelope glycoprotein or by inserting soluble adaptors between the viral particle and the desired target cell. Proof of principle for this type of technology has been provided in the past for both oncoretroviral and lentiviral vectors. However, increasing specificity, while maintaining infectivity of a retroviral particle, turned out to be difficult and the entire technology is still far from meeting the safety and efficacy requirements for real clinical application (reviewed in Larochelle et al., 2002). On the other hand, redirecting transcriptional properties has proven a relatively easier task, although the RNA nature and the limited size of a retroviral genome impose significant constraints on the complexity of the regulation that can be achieved in both oncoretroviral and lentiviral vectors.

The goal of this project is the development and the exploitation of transcriptionally-targeted lentiviral vectors, based on the use of cellular transcription control elements conferring tissue- and/or lineage-specific expression upon integration into the target cells. This approach will be validated in the context of gene transfer and therapy models requiring stem cells transduction and restriction of gene expression to specific progeny differentiating from transduced cells. The project implies: 1) the design of new vectors and the development of suitable, high-titer packaging systems; 2) the transduction of cell lines and stem cells of different origin and the analysis of specifically restricted transgene expression *in vitro*; 3) the development of *in vivo* models to test the efficacy of the newly designed vectors in restricting the expression of a given transgene in a tissue- and cell-specific fashion and, finally, 4) the development of transcriptionally-targeted lentiviral vector for β -globin expression.

1.6 Specific Background

Retroviral vectors are widely used to integrate and express exogenous genes into a variety of animal cells and provide a safe and relatively efficient gene transfer tool for human gene therapy (Verma and Somia, 1997). To date, they represent the only gene transfer system allowing active integration of foreign DNA at high efficiency into the target cell genome and are the vectors of choice in most of the clinical trials for gene therapy. However, a significant limitation of retroviral vectors derived from the Moloney murine leukemia virus (MoMLV) backbone is their inability to integrate into the genome of non-dividing cells, which essentially limits their use to *ex vivo* applications and to cells which can be induced to replicate in culture without losing a specific, therapeutically relevant phenotype. As an example, hematopoietic stem cells (HSCs) are very difficult to maintain in culture in an active mitotic state without compromising their self-renewing and bone marrow (BM) repopulating capacity and are therefore difficult to transduce with retroviral vectors at an efficiency compatible with clinical applications. A number of groups have recently reported that HIV-1-derived LV vectors can integrate into the genome of non-dividing cells *in vivo* and *ex vivo* and are much more efficient than MoMLV-derived vectors in transducing HSCs without compromising their repopulation capacity upon transplantation (Miyoshi et al., 1999; Woods et al., 2000). Several years of research have drastically improved both design and packaging of LV vectors, and virtually abolished the safety concerns originally raised by the idea of transducing human cells with a vector derived from HIV.

For correction of genetic disorders affecting a specific progeny of pluripotent stem cells, the ultimate goal of gene therapy would be to integrate a therapeutic gene in long-lasting and self-renewing stem cells and regulate its expression in specific, differentiated lineages. In these applications, controlling the expression of a transferred, therapeutic gene at quantitative and qualitative (tissue- or cell-specific) levels is a critical and often limiting, factor. Appropriate transgene regulation in the framework of a retroviral vector is a difficult task, due to a number of technical factors such as limited vector capacity, transcriptional interference between the viral LTR and internal enhancers/promoters and genetic instability of complex regulatory sequences in this context (Coffin et al., 1997). Transcriptional targeting of LV vectors meets essentially the same type of technical difficulties encountered with oncoretroviral vectors. However, transcriptional interference is usually not a problem in a lentiviral context, since the “self-inactivating” (SIN) design is mandatory to minimize the regions of

potential overlapping, and therefore recombination, between the transfer vector and the packaging construct (see Section 1.2.3.4). This allows the use of internal promoters combined with tissue- or cell-specific enhancers to regulate transgene expression. With this simple design, expression of a reporter or a therapeutic transgene has been effectively restricted to erythroblasts and to antigen-presenting cells derived from murine or human HSCs, by the use of erythroid-specific enhancers driving the ankyrin-1 promoter or the human HLA-DRA promoter respectively (Cui et al., 2002; Moreau-Gaudry et al., 2001; Richard et al., 2001). Attempts have also been made to introduce into LV vectors exogenously regulated transcriptional elements, such as a tetracycline-responsive element linked to a minimal promoter to drive the gene of interest and different variants of tetracycline-regulated, chimeric transcriptional activators (Kafri et al., 2000; Vigna and Naldini, 2000). The major limitation of using internal transcription units within LV vectors is the absence of introns in the sub genomic transcript used to express the gene of interest, which significantly affects its post-transcriptional fate (polyadenylation, nuclear export, stability) and ultimately reduces its efficacy in terms of protein output. For some gene therapy applications, for example, the correction of globin chain imbalance in β -thalassemia, a high protein output is just as important as its restricted expression, which makes practically inadequate the use of intronless β -globin cDNAs expressed by internal promoters. This problem has been partially resolved by placing the entire human β -globin gene under the control of its own promoter and a reduced version of the β -globin LCR in reverse orientation within a first-generation LV vector. With this design, potentially therapeutic levels of human β -globin have been obtained for the first time in a murine model of β -thalassemia (May et al., 2000; May et al., 2002; May and Sadelain, 2001; Rivella et al., 2003).

As in the case of oncoretroviral vectors, transcriptional targeting of LV vectors can be conveniently achieved by LTR enhancer replacement (Lotti et al., 2002). A critical advantage of this targeting strategy is the use of the spliced, major viral transcript to express the gene of interest, which partially overcomes the major limitation of the internal promoter design. The internal, less efficient transcription unit can nevertheless be used to independently express a second gene providing an additional function, such as an *in vivo* selectable marker. An additional advantage of LTR enhancer replacement as a targeting strategy is that the integrated provirus carries two copies of a genomic enhancer within the two LTRs. The presence of two active enhancers flanking the transgene transcriptional unit could reduce the chances of chromatin-mediated

inactivation of transcription, which is known to affect the long-term maintenance of retroviral transgene expression *in vivo*, particularly in stem cells (Challita and Kohn, 1994). Double copies of at least some cell-specific enhancers could indeed act as transcriptional insulators, thus replacing LCRs, matrix-attachment or other elements influencing chromatin configuration to shield randomly integrated transgenes from chromatin-mediated silencing. Indeed, reduced LCR sequences do not appear to confer position-independent expression to the viral-encoded transgene [May, 2000 #285], and their role in maintaining open chromatin conformation to transcribed loci has been challenged by several authors (Epner et al., 1998; Reik et al., 1998). Matrix- or scaffold-attachment elements have also been introduced in retroviral vectors to reduce position effect variegation, with only partial success (Dang et al., 2000; Park et al., 2000). Binding of tissue- or lineage-specific transcription factors to specific regulatory regions (e.g., those defined by DNase hypersensitive sites) could be a key factor in initiating and maintaining active chromatin structures around genes regulated in development or differentiation (Martin et al., 1996; Orkin, 1995) providing a rationale for using transcriptionally targeted vectors based on specific enhancers to restrict transgene expression to a specific progeny of the HSC.

Gene therapy of hemoglobinopathies or thalassemias would require restricted expression of a globin transgene only to the erythroid progeny of hematopoietic stem cells and represents one of the most challenging models to test the efficiency of transcriptional targeting of viral vectors. The β -thalassemias are a group of inherited anemias characterized by reduced or absent production of hemoglobin β -chains (see Section 1.3.2.1). Conventional treatment is based on lifelong blood transfusion and continuous iron chelation. The only available cure is allogeneic bone marrow transplantation (BMT), which is however available for less than 30% of the thalassemic patients. Gene therapy could be a potential alternative to BMT. The clinical history of the disease and over 15 years of BMT experience indicate that even a mild correction (>15%) of the globin imbalance in a fraction (>25%) of maturing erythroblasts is sufficient to reduce the morbidity caused by ineffective erythropoiesis, to improve the clinical management of the disease and to increase the patients' life expectancy. For therapeutic purposes, reproducing the full complex regulation of the β -globin gene might therefore be unnecessary. During the years, a number of scientists have tried to obtain lineage-restricted, high-level transcription of the β -globin gene in the framework of a MoMLV-derived vector, with limited success (Sadelain et al., 1995). Recently, the

synthesis of potentially therapeutic levels of human β -globin was achieved in the erythrocytes of bone marrow chimeras transplanted with β -thalassemic bone marrow cells, transduced with the 1st generation LV vector TNS9 (May et al., 2000). TNS9 lentiviral vector contains the human β -globin gene, including extended promoter sequences and the β -globin 3' proximal enhancer, and LCR elements (3.2kb), cloned in a reverse orientation in respect to the HIV LTR transcriptional direction. Although these results showed that appropriate regulation of a globin gene can be technically achieved, intrinsic limitations to this system are the lacking of position-independent expression of virally encoded β -globin, demonstrated by clonal analysis of transduced MEL cells (May et al., 2000), and the size of the vector, which is unlikely to allow the insertion of a second transgene for selection of transduced stem cells. Moreover, the TNS9 vector has been done in the backbone of a first-generation lentiviral vector, and it contains two intact HIV-1 LTRs. A SIN version of the TNS9 lentiviral vector must be developed using the third-generation packaging system to prevent recombination events generating replication-competent HIV-1 virus.

Chapter 2

MATERIAL AND METHODS

2.1 Reagents-suppliers

2.1.1 Chemicals

All chemicals were obtained from FLUKA, Sigma Chemical Co. or Roche.

Other chemicals used during the study were obtained from the following sources:

Agarose for gel electrophoresis	FMC
DNA markers	Gibco Brl
Protein markers	Gibco Brl
X-Ray film	KODAK
Hydrolysed milk powder	Nestle
Nitrocellulose for western blot	Schleicher and Schuell
Nylon membrane for Southern/Northern blot	Hybond
ELISA p24	NUNC
FICOLL	Nycomed

2.1.2 Radiochemicals

$\alpha^{32}\text{P}$ dCTP

2.1.3 Enzymes

All DNA modification enzymes and buffers were received from Roche.

2.1.4 Restriction enzymes and buffers

All restriction enzymes and buffers were obtained from either Roche or New England Biolabs.

2.1.5 Bacterial strains and growth

The DH5 α bacterial strain (F', hsdR17, $r_k^-m_k^+$, recA1, endA1) was used for all transformations, and grown in Luria Broth media prepared within the Institute.

2.1.6 Plasmids

Creation of other plasmids are described below (Section 2.5.1). The following were provided by Prof. Luigi Naldini (University of Turin Medical School):

- the pCMVΔR8.91 packaging vector.
- pRRL.sin-18.CMV.GFP, pHR II
- the pMD.G plasmid encoding VSV.G protein.

Unless otherwise stated, all the plasmids contained the ampicillin resistance gene allowing selective growth in LB media.

2.1.7 Solutions and buffers

Hematopoietic cell buffer for flow cytometric analysis (HC FACS):	PBS containing 0.1 % (weight for volume) ratio (w/v) Sodium azide, 0.3 % w/v BSA
Standard Cell lines buffer for flow cytometric analysis:	PBS containing 5 % w/v FCS
50x TAE for agarose gel (for 1 litre)	242g Tris Base 57.1 ml Glacial acetic acid 100ml 0.5M EDTA, pH8.0 Water to 1 litre

2.2 Tissue culture reagents

2.2.1 Plastic ware

All standard tissue culture plastic ware, (tissue culture flasks, roller bottles, collagen coated transwell apparatus, tissue culture plates) was obtained from Corning Costar.

2.2.2 Media

IMDM	Biowhittaker
X-VIVO 10	Biowhittaker
RPMI 1640	Euroclone
BIT 9500	Stem Cell technology
DMEM	Euroclone

2.2.3 Sera, Supplements and antibiotics

Fetal Calf Serum	Mascia Brunelli, Italy
Fetal Bovine Serum	Euroclone
Trypsin	Gibco Brl
L-Glutamine	Gibco Brl
Penicillin/Streptomycin	Gibco Brl
HEPES	Gibco Brl
Polybrene	Sigma

2.2.4 Growth factors

Recombinant human Stem cell factor (SCF)	R&D Systems, and Pepro Tech, England
Recombinant human Flt-3 Ligand (Flt3-L)	Pepro Tech, England
Recombinant human Thrombopoietin (TPO)	PeproTech, England
Recombinant human interleukin-3 (rhIL-3)	R&D Systems
Recombinant human interleukin-6 (rhIL-6)	R&D Systems
Erythropoietin (EPO)	R&D Systems

2.2.5 Other

Phosphate buffered Saline (PBS) was obtained from Euroclone.

2.3 Antibodies and antisera

Anti human NGF antibody:

Hybridoma supernatant produced at DIBIT HSR.

Anti human NGF biotin conjugated:

Antibody purified from hybridoma and conjugated with Biotin, by facility in DIBIT HSR.

Antimouse IgG (FITC, R-PE conjugated):

Obtained from Southern Biotechnology Associates, U.S.A.

Mouse IgG (used for block in Hematopoietic gene transfer analysis):

Obtained from Sigma Chemical co. U.S.A.

Streptavidin conjugated with R-PE, FITC, or Tricolor:

Obtained from Caltag Laboratories, U.S.A.

Anti-human CD38, R-PE, FITC, or Tricolor conjugated:

Obtained from Caltag laboratories, U.S.A.

Anti-human CD13 antibodies , R-PE conjugated:

Obtained from Caltag laboratories, U.S.A.

Antihuman CD45 antibodies , R-PE conjugated:

Obtained from Caltag laboratories, U.S.A.

Antihuman GpA antibodies , R-PE conjugated:

Obtained from Caltag laboratories, U.S.A.

Anti human CD34 antibodies (anti HPCA-2), R-PE or FITC conjugated:

Obtained from Becton Dickinson Immunocytometry systems, U.S.A.

Anti human CD45, R-PE, FITC, or Tricolor conjugated:

Obtained from Caltag laboratories, U.S.A.

Isotype control antibodies. Mouse IgG conjugated with R-PE, FITC, or Tricolor:

Obtained from Caltag laboratories, U.S.A.

Anti-mouse Ter 119-PE:

Obtained from Pharmigen

Anti-mouse Gr-1-PE:

Obtained from Pharmigen

Anti-mouse CD.45.1-FITC/-TC:

Obtained from Pharmigen

Anti-mouse CD.45.1-PE:

Obtained from Pharmigen

Anti human globins:

Obtained from ICN Biotechnology, U.S.A.

Anti human-FLAG:

Obtained from Sigma.

2.4 Cells

2.4.1 Stable cell lines

<u>Name</u>	<u>Description</u>	<u>Source</u>
293T	Endothelial, Human embryonic kidney	Naldini L, University of Turin Medical School
NIH 3T3	Fibroblast, murine bone marrow	Ferrari G., TIGET
HeLa	Human carcinoma	From ATCC
HEL	Human erythroleukemic	From ATCC
K562	Human myeloblastic	From ATCC
U937	Human monocytes	From ATCC
MEL	Murine erythroleukemic	From Antoniou M.
KASUMI-1	Human lymphoblastic	From ATCC

2.4.2 Primary cells

Human hematopoietic cells:

All primary human cells were received from Ospedale San Raffaele, Milan, Italy, with informed consent, following the legal and ethical regulations instated by both the above mentioned hospital and by the Italian Fisica Sanitaria (Rome, Italy).

Human CD34⁺ cells:

Were most kindly purified and prepared from UCB, BM or MPB sources by either Claudia Rossi, by FICOLL density separation followed by magnetic selection based on the Miltenyi MACS kit for cell separation, following the manufacturers recommended protocol. Samples were frozen in FCS containing 10 % DMSO, and stored in liquid Nitrogen until required for use.

2.5 Methods

Methods are divided into 4 sections:

2.5.1 Molecular methods

2.5.2 Tissue culture methods

2.5.3 Gene transfer methods

2.5.4 Animal study methods

2.5.1 Molecular methods

2.5.1.1 Generation of erythroid specific LV vectors:

An *EcoRI/SacI* fragment containing part of the 3' LTR of the HIV-1 derived lentiviral vector pHR2 was sub-cloned into the *PvuII* site of the pUC-19 plasmid. The plasmid was digested with *EcoRV* and *BanII*, self-ligated to generate a -418 to -40 deletion (where +1 is the transcription start site) in the U3 region of the LTR, or ligated with a 200-bp *BamHI* fragment containing the GATA-1 HS2 human genomic fragment (-856 to -655) to obtain a chimerical LTR. The two LTRs were then introduced in the pRRL.SIN vector backbone to obtain the pRRL.sin-40.GFP (-40) and the pRRL.GATA.GFP (G) vectors, respectively. A second, independent transcriptional cassette (PGK. Δ LN Δ GFR) was introduced into the *HindIII/SacII* sites to generate the vectors pRRL.sin-40.GFP.PGK. Δ N (-40/P) and pRRL.GATA.GFP.PGK. Δ N (G/P). The woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) was

cloned in the filled *EcoRI* or *HindII* sites of the G/P vector to generate the pRRL.GATA.GFP.W.PGK.ΔN. (G.W/P) or pRRL.GATA.GFP.PGK. ΔN.W (G/P.W) vectors, respectively. To generate the pRRL.sin-18.GATA.GFP (-18/G) vector, a fragment of 370 bp containing the GATA-1 HS2 and a portion of the HIV-1 LTR (-40 to +75) was inserted in the filled *BamHI* site of the pRRL.GFP.sin-18 vector. For some experiments, the G.W/P vector was further modified by the insertion of a 118-bp, *HpaII/ClaI* fragment containing the HIV-1 central polypurine tract (cPPT), as previously described (Follenzi et al., 2000).

β-globin vectors:

The FLAG sequence was inserted at the 5' end of the human β-globin coding sequence by PCR.

We used as template the pT7T3D plasmid containing the human beta globin cDNA and the two primers:

primer forward: 5' ccccatggactacaaggacgacgacgacaaggtgcacctgactcctgaggag 3'

primer reverse: 5'gggcggccgcaaggaagcgagccttagtgatacttggtggcc 3'.

The PCR product was digested *NcoI/NotI*, blunted and cloned into pBS.II.SK digested with *EcoRV*. The vector containing the insert in opposite orientation was digested *XmaI/SalI*, and the insert was cloned in the two lentiviral vectors *pRRL.sin-40.GATA1.ppt.GFP.wpre* and *pRRL.sin-40.GATA1.ppt.GFP.wpre.PGK.ΔLNGFR* digested *AgeI/SalI*. In this way the GFP sequence was replaced by FLAG-β-globin and we obtained the two final vectors: *pRRL.GATA1.βglo* and *pRRL.GATA1.βgloPGK.ΔN*.

2.5.1.2 Transformation of competent bacteria and small scale bacteria preparations

DNA to be transformed was mixed with 200μl of competent DH5α cells prepared specifically for the heat shock mediated transformation method (Maniatis, Fritsch *et al*, 1982). The mix was incubated on ice for 20 minutes, then 1 minute and 45 seconds in a waterbath at 42°C, and then returned to ice for a further 10 minutes. The mix was then added to 1 ml of LB media and incubated at 37°C with agitation for one hour. The mix was then plated onto LB agar plates, containing the required antibiotic resistance and placed overnight in a 37°C bacterial incubator. The following day, colonies were picked and placed into 1.5 ml of LB media containing antibiotics, and grown overnight with

agitation at 37°C. The small bacteria preparations were subjected to small or large scale DNA preparation as required.

2.5.1.3 Small scale (miniprep) preparation of plasmid DNA from bacteria

Small amounts of plasmid DNA were purified from bacteria, for restriction analysis, following the alkaline lysis method for DNA isolation (Maniatis, Fritsch *et al*, 1982).

2.5.1.4 Large scale preparation of plasmid DNA

This method is a scaled up method of the alkaline lysis method, using identical solutions unless stated. Typically 500 ml of an overnight growth of DH5 α bacteria in antibiotic containing LB media was used per preparation. The bacteria were pelleted at 6000 rpm for 10 minutes in a Sorvall centrifuge. The residual media was discarded and the centrifuge tube inverted to drain as much media as possible away from the pellet. 4 ml of alkaline lysis solution 1, containing Lysozyme at a final concentration of 12.5 μ g/ml, was added to the pellet, and the bacteria resuspended by gentle mixing. The bacterial mix was then placed on ice for 10 minutes. 8 ml of alkaline lysis solution 2, was then added, the sample mixed and returned to ice for a further 10 minutes. 7 ml of ice cold alkaline lysis solution 3 was added, the sample again thoroughly mixed, and returned to ice for a further 20-30 minutes.

The tube was centrifuged at 11000 rpm in a Sorvall centrifuge, the lysate filtered through medical gauze into pyrex tube (the residual pellet was discarded) and 11ml of isopropanol added to precipitate the nucleic acid. 10 minutes later the samples were centrifuged at 11000 rpm, the liquid discarded, the pellet properly drained, and then resuspended in 2.5 ml of DNase free water. RNase was added to a final concentration of 2 μ g/ml and the sample incubated at 37°C for 15 minutes.

Beckman quick seal tubes (BQS) were prepared as follows. 11g of Caesium Chloride (CsCl) were measured into 50 ml Falcon tubes to which was added 400 μ l of Ethidium Bromide (10mg/ml stock). 7.5 ml of water was then added to the RNase treated pellet, and this was added to the CsCl. After the CsCl had dissolved, the solution was taken up using a 10 ml syringe and injected into the BQS, the tubes balanced, and then sealed using the Beckman heat sealing device.

Samples were centrifuged in a Beckman NVT 65 rotor at 55,000 rpm, for at least 20 hours. The tubes were gently removed from the rotor, a hole punched in the top of the tube using a large gauged needle, and a syringe with needle attached was inserted just

below the DNA band which was clearly distinguished from the rest of the gradient due to the intense red colour following the ethidium bromide intercalation. The DNA band was gently removed and injected into a 15 ml Falcon tube. 1 ml of butanol saturated with water was added to the tubes, the tubes shaken, left to settle and the upper phase discarded (clearly pink coloured). This process was repeated several times until no pink colour was visible. The process was then repeated one more time after this to be sure of ethidium bromide removal.

An equal volume of isopropanol saturated with CsCl was added, the tubes again mixed left to settle and the upper phase discarded. This process was repeated twice. Water was then added to the tubes, up to 5 ml, 250 ml of 3M sodium acetate added, and a further 10 ml of absolute ethanol added on top. Tubes were mixed and placed at -20°C for 30 minutes and then washed by repeated 70% ethanol mixing and centrifugation. Purified DNA was resuspended in DNase free water and quantified using the standard 260/280nm absorbance method on a spectrophotometer. DNA was stored at 4°C if required immediately, or stored at -20°C for longer use.

2.5.1.5 Southern blot analysis

Extraction of genomic DNA from cells:

$1-5 \times 10^6$ cells were lysed in 800 μl the following buffer:

Southern Lysis buffer:

50 mM Tris pH8.0
100mM EDTA
400mM NaCl
1% w/v SDS

25 μl of proteinase K (10mg/ml stock) was added and the mix incubated overnight at 55°C .

100 μg of RNase was added and the mix incubated for a further 2 hours at 37°C . An equal volume of phenol/chloroform was added and the samples mixed for 15 minutes, followed by centrifugation for 30 minutes at 15,000 rpm. The aqueous phase was removed and an equal volume of phenol/chloroform added. The above step was performed, the aqueous phase taken and an equal volume of chloroform added, the samples mixed and centrifuged as above, the aqueous phase taken and nucleic acid precipitated by the addition of an equal volume of isopropanol.

A glass pasteur pipette was blunted by inserting into a bunsen flame and the precipitated DNA extracted by dipping the pasteur and gently pulling. The genomic DNA was clearly visible, it was allowed to air dry briefly, and then the inserted into 100 µl of DNase free water, and the DNA allowed to dissolve overnight at 4°C. Nucleic acid concentration was determined using the 260/280nm absorbance measurements on a spectrophotometer.

Isolation of probe:

20µg of the pEGFP-C3C (ClonTech), DNA were digested with the enzymes, *Eco47III* and *ScaI*, to release a 790bp sequence of GFP gene. After running on a 0.8% w/v agarose gel, in TAE buffer, the band corresponding to the required DNA was excised using a scalpel, and the DNA fragment purified using the QiaexII DNA extraction kit from Qiagen following the manufacturers instructions. Concentration was measured and the probe labelled as described below.

Digestion of genomic DNA and gel electrophoresis:

10 µg of genomic DNA was digested with *EcoRI*, *AflIII* or *XbaI* enzyme by repeated addition of enzyme and buffer, (2 separate additions of concentrated enzyme and buffer over 16-18 hours). The digested DNA was run on a 3% agarose gel in TAE buffer, containing ethidium bromide, overnight at 35 volts. The gel was photographed.

Southern blotting:

The DNA was denatured by incubating the gel in 0.2M NaOH, 0.6M NaCl for 1 hour with one exchange of buffer. The gel was then neutralised by incubating the gel for 1 hour in 1.0M Tris HCl, pH7.5, 1.5M NaCl. Southern blot assembly performed as described (Maniatis, Fritsch *et al*, 1982) using Hybond Nylon filters (presoaked in distilled water and 10X SSC. Southern blot was performed overnight in 10X SSC. After blotting the filter was rinsed in 6X SSC and crosslinked using a Stratalinker.

Hybridisation:

The filter was washed with 0.1X SSC, 0.5% w/v SDS at 65°C for 1 hour, exchanging the buffer, twice, followed by an overnight incubation in prehybridisation solution at 42°C.

Pre-hybridisation solution:

5X Denhardt's
5X SSCP
50% deionised formamide

The prehybridisation solution was removed and replaced with hybridisation solution containing the radioactive labeled probe. The filter was incubated overnight at 50°C, followed by a 30 minute wash in 2X SSC, 0.1% SDS (with one change) and a 2 hour wash at 60°C with 0.1X SSC, 0.1% w/v SDS (with 3 changes). The filter was blot dried and exposed to KODAK autoradiograph film, for various times and the films developed.

Radioactive labelling of probe:

Radioactive labeling was performed using the Roche Random primed DNA labeling kit and the quick spin columns, following the manufacturers instructions.

Southern blot solutions:

100X Denhardts:	1g FICOLL 400 1g Polyvinylpropylene(PVP) 1g BSA (fraction V) Up to 50 ml with water
20X SSC	350.6g NaCl 176.5g Citric acid, trisodium salt DNase free distilled water up to 2 litres
20X SSCP	88.2g Sodium citrate (trisodium salt) 140.3g NaCl 43.7g Na ₂ HPO ₄ 12.7g Na ₂ HPO ₄ ·H ₂ O

	DNase free distilled water up to 1 litre
Hybridisation solution (for 20 ml):	6 ml 20X SSC 0.2 ml 100X Denhardtts 10 ml deionised formamide 1 ml 10% w/v SDS 2g Dextran Sulphate 2.8 ml of B.S256+probe
B.S256(10X)	16 mg polyA 16 mg polyC 400 mg yeast t-RNA 100 mg Salmon Sperm DNA 9 mg <i>E. coli</i> DNA

Note: The radioactive probe is mixed with B.S256, boiled for 10 minutes and quenched on ice, prior to adding to hybridisation solution. This is to ensure that the probe is denatured.

2.5.1.6 Northern blot analysis

Total cellular RNA was extracted by guanidine-isothiocyanate, poly(A)⁺ selected by oligo(dT)-cellulose chromatography, size-fractionated on 1% agarose-formaldehyde gel, blotted onto nylon membranes, hybridized to 10⁷ dpm of ³²P-labeled NGFR, GFP, β -globin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probes, and exposed to X-ray film.

Pre-hybridization solution per Northern	20x SSCP Denhardtts 100x SDS 10 % Salmon sperm Formamide D Water
Hybridization solution per Northern	20x SSCP Denhardtts SDS 10%

	Formamide D Dextran sulphate 50 %
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2.5.1.7 Poliacrylamide gel electrophoresis (PAGE) and western blotting

PAGE:

12% denaturing PAGE gels were made following the published instructions (Maniatis, Fritsch *et al*, 1982), and assembled on the Biorad miniprotein II gel apparatus following the manufacturers recommended instructions.

1-5x10⁶ cell samples, were first lysed in 200 µl of protein lysis buffer.

Protein lysis buffer:	150 mM NaCl 1% v/v Nonidet P-40 0.5% w/v DOC 0.1% w/v SDS 50 mM Tris (pH8.0)
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25 µl of lysate was mixed with 25 µl of 2 fold concentration cracking buffer containing β-Mercaptoethanol, and loaded into the well of the PAGE using a Hamilton syringe.

2 fold cracking buffer (for 10 ml):	1.5 ml of 0.83 M Tris Hcl pH 6,8 3 ml of 20% SDS 2 ml of glycerol 1ml of β-Mercaptoethanol 2.5 ml of water 250 mg of Bromo Phenol Blue
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The gel was run in Tris-Glycine buffer (27.9 g glycine, 6 g Tris, dissolved in 1 litre of water), containing 0.1% v/v SDS, at 55 mA, until the Bromo Phenol Blue dye had run out of the gel. As a molecular weight marker, 4 µl of Rainbow marker (Biorad) was used.

Blotting:

Nitrocellulose membranes (Sleicher and Schuell) cut to the same size as the PAGE gel were pre-soaked in transfer buffer:

Transfer buffer (for 1 litre):	3g Tris 14.4 g Glycine 200 ml Methanol 800 ml Water
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Blotting was performed using the Biorad western blotting apparatus and assembled following the manufacturers instructions. Upon assembly, the inside of the apparatus was filled with transfer buffer, and run overnight at 200 volts at 4°C.

Antibody staining:

After transfer of the proteins had been performed, the membrane was blocked in PBS containing hydrolysed milk (2,5% w/v) for 30 minutes. The mouse anti-FLAG or the rabbit anti-human globin antibody (ICN Biotechnology, U.S.A) was added to the blocking reaction at a final concentration of 10 µg/ml and incubated with agitation for 2 hours at room temperature. The membrane was washed three times with PBS/0.05% v/v Tween 20, and fresh blocking solution containing the anti-mouse or anti-Rabbit IgG Peroxidase-conjugated antibody at a 1 in 10,000 dilution was added to the membrane. Incubation was performed for two hours at room temperature or 16 h at 4°C with agitation. The membrane was washed as before and peroxidase activity was measured using the commercially available ECL kit (Amersham) following the recommended instructions. The membrane was exposed to KODAK autoradiograph film and developed.

2.5.1.8 Polymerase chain reaction specific for GFP:

Primers specific for the GFP were obtained from PRIMM, Italy and had the following sequences:

GFP (sense): 5' -atg gtg agc aag ggc gag-3'.

GFP (antisense): 5' -gtt act tgt aca gct cgt c-3'

Both primers had melting temperatures of 55°C. PCR with these primers enables distinction from endogenous NGFr sequences, as these have been designed against the cDNA.

dNTP's were obtained from Roche, water was in house prepared DNase free, while the Taq polymerase enzyme and buffer were obtained from Dynazyme.

Cell lysates to serve as templates for the PCR reaction were prepared by lysing 100,000 cells in 25 µl of lysis buffer (1:1 mix of lysis buffer A and B, containing Proteinsase K at a final concentration of 50µg/ml):

Lysis buffer A	100mM KCl 10 mM Tris pH8.3 2.5mM Mg Cl ₂
Lysis buffer B	10 mM Tris pH8.3 2.5 mM MgCl ₂ 1% v/v Tween 20 1% v/v Nonidet P-40

Lysed cells were incubated overnight at 37°C, heat-inactivated for 15 minutes at 94°C and then stored at -20°C until required.

The PCR mix was prepared as follows:

GFP-F (25pmol/ml)	1 µl
GFP-R (25pmol/ml)	1 µl
10x Taq buffer	5 µl
Taq polymerase	1 µl
dNTP(10mM)	1 µl
Lysate	25 µl
Water	Up to final volume of 50 µl

The mix was placed in a Perkin Elmer PCR tube, 25 µl of mineral oil placed on top, and PCR performed in a Cellbio Hibaid Touchdown PCR machine following the programme below:

A first denaturation of 5 minutes at 95°C then a cycle platform composed of:

- Denaturation step, of 45 seconds, 95°C
- Annealing step, of 2 minutes at 60°C
- Extension step, of 3 minutes at 72°C

The samples were subjected to 50 cycles, followed by a final 10 minutes extension at 72°C.

PCR samples were mixed with DNA loading dye and run on a 0.8% w/v agarose gel in TAE buffer, containing Ethidium Bromide, and visualised on a UV lamp.

Negative controls for the PCR reaction were water alone. The positive control dilution series was performed by diluting the positive control cell lysate in lysis buffer.

2.5.2 Tissue culture methods

2.5.2.1 Maintenance of cell lines

HeLa, MEL and NIH-3T3 were maintained in DMEM media supplemented with 10% FCS IMDM media containing 10% FBS. HEL, K562, U937 and KASUMI were maintained in RPMI-1640 with 10% FCS. 293T cell lines were maintained in IMDM media and 10% FBS. All these media were supplemented with penicillin/streptomycin at 100 units/ml, and L-glutamine at 2 mM (complete media). Cells were typically passaged twice a week by washing once in PBS, adding trypsin solution to the adherent cells, incubating for 5 minutes and then resuspending in fresh media. Non-adherent cells were passaged by simple dilution of the cells in fresh media. Typical passage levels are described below.

All adherent cell lines were passaged at 1/5 to 1/20 dilutions twice per week depending on experimental requirements.

K562, HEL, MEL, KASUMI-1 and U937 were passaged at 1/10 dilutions twice a week. All cells were maintained at 37°C in a humidified incubator with atmospheric 5% CO₂.

2.5.3 Gene transfer methods.

2.5.3.1 Generation of Lentiviral vectors:

Viral stocks pseudotyped with the vesicular stomatitis G protein (VSV-G) were prepared by transient co-transfection of 293T cells using a three-plasmid system (the transfer vector, the pCMVΔR8.74 encoding Gag, Pol, Tat and Rev, and the pMD.G plasmid encoding VSV-G).

Transfection of DNA using Calcium phosphate precipitation:

1. Seed and incubate 5×10^6 293T cells in a 10 cm dish, approximately 24 hours before transfection in IMDM, 10%FBS, Penicillin (25U/ml), Streptomycin (25U/ml).
2. Change medium 2 hours before transfection.
3. The plasmid DNA mix is prepared by adding 3,5 μ g ENV plasmid (pMD2-VSV-G), 6,5 μ g of Core Packaging plasmid (pRRLsin18.PPT.CMV.GF.Wpre) together per dish. The plasmid solution is made up to a final volume of 450 μ l with 0.1XTE/dpH₂O (2:1) in a 50 ml polypropylene tube. Finally add 50 μ l of 2.5M CaCl₂.
4. The precipitate is formed by dropwise addition of 500 μ l DNA-TE-CaCl₂ mixture from step while vortexing at full speed. The precipitate should be added to the 293T cells immediately following addition the 2X HBS. High magnification microscopy of cells should reveal a very small granular precipitate of the CaPi-precipitate plasmid DNA, initially above the cell monolayer, and after incubation in the 37 °C incubator overnight, on the bottom of the plate in the spaces between the cells.
5. The CaPi-precipitated plasmid DNA should be allowed to stay on the cells for 14-16 hours, after which the media should be replaced with fresh media for virus collection to begin. Discard medium as infectious waste.
6. Collect the cell supernatans at 25 and 48 hours after changing the media.
7. Centrifuge a 1000 rpm, for 5 min at and filter through 0.22 μ m pore nitrocellulose filter.
8. Use the conditioned supernatant as it is or proceed to concentration.

2.5.3.2 Viral harvest and concentration

1. Collect the supernatant from calcium phosphate-transfected 293T packaging cells.
2. Centrifuge at 1500 rpm for 5 min at RT and filter through 0.22 μ m filter.
3. Use the conditioned supernatant as it is or proceed to concentration by ultracentrifugation.
4. Concentrate the conditioning medium by ultracentrifugation at 50,000 x g (19,500 rpm in SW28 rotor) 2 h at RT.
5. Discard the supernatant by decanting and re-suspend the pellets in 0.5 ml of sterile PBS containing 1% BSA.
6. Store at 4°C the concentrated vector from the first collection until the end of the second collection.

7. Fill the tubes used for the first collection with the supernatants from the second collection.
8. Repeat points 1-2-3 for the second collection.
9. Re-suspend the final pellets in a very small volume (1/500 of the starting volume of medium) of sterile PBS-1% BSA.
10. Pool in a small tube and rotate on a wheel at RT for 1 h.
11. Split into small aliquots (20-50 μ l), store at -80°C and titer after freezing.

2.5.3.3 Titration of Lentiviral vectors:

1. Plate 5×10^4 HeLa cells per well a day before in a 6-well COSTAR (the day after you will have approximately 10^5 cells/well).
2. The following day, prepare serial ten-fold dilutions of viral stocks. Take a 24-well plate and add in each well 1.8 ml of medium (IMDM 10% FBS). Then add to the first well of the first row 200 μ l of viral stock (10^{-1} dilution). After pipetting several times, change tip and take 200 μ l of 10^{-1} dilution and put in the second well. Take 200 μ l of 10^{-2} dilution and put in the third well and continue until you have 10^{-6} dilution.
3. If you are titrating concentrated vector stock, you should add 2ml of IMDM in the first well of the dilution plate and 2 μ l of the concentrated vector preparation and continue with the serial dilutions as described above (in this case you have dilutions from 10^{-3} to 10^{-8}).
4. Take HeLa cells from the incubator and aspirate the media. Add 1ml of medium (IMDM 10% FBS) with 2X polybrene (1000X-8mg/ml).
5. Add to wells 1 ml of serial dilutions changing tips each time or starting from the most diluted sample.
6. Incubate for 48-72 hours at 37°C in a 5% CO_2 incubator.
7. Wash wells with 2 ml of PBS.
8. Add 200 μ l of trypsin in PBS to each well. Wait until cells are detached from plates (5' at 37°C).
9. Add 2 ml of PBS to each well and harvest cells in FACS tubes.
10. Centrifuge at 100 rpm for 5 min at RT.
11. Aspirate the supernatant and add 1 ml of FIXING SOLUTION to the pellet, vortex the tubes (samples are stable at 4° for few days).
12. FACS samples and calculate the titer.

Notes:

The MOI (multiplicity of infection): TU/ml/n. of cells to be infected for the following ranges: infection efficiency is primarily controlled by vector concentration (TU/ml). MOI should be considered only for certain ranges of concentration of cells ($>10^4$ cells/ml) and vector ($>10^6$ TU/ml).

2.5.3.4 Stable cell lines.

1. HeLa and NIH-3T3 cells were maintained in DMEM supplemented with 10% FCS, and transduced in six-well plates in the presence of 8 $\mu\text{g/ml}$ of polybrene.
2. Human erythroleukemic (HEL, MEL), myeloblastic (K562) and monocytic (U937, Kasumi-1) cell lines were grown in RPMI 1640 supplemented with 10% FCS, and transduced with viral supernatant at different MOI in the presence of 4 $\mu\text{g/ml}$ of polybrene.
3. On day 4 after transduction, cells were harvested and analyzed by flow cytometry for transgene expression.

2.5.3.5 CD34⁺ stem/progenitor cells.

1. Human CD34⁺ stem/progenitor cells were purified from the Ficoll mononuclear cell fraction of umbilical cord blood by positive selection using the CD34 magnetic cell isolation kit.
2. CD34⁺ cells were infected with viral stocks at a MOI of 50-200 in serum-free IMDM containing 20% BIT serum substitute, 20 ng/ml rhIL-6, 20 ng/ml rhTPO, 100 ng/ml stem cell factor and Flt3-L, for 24 hours.
3. Transduced CD34⁺ cells were washed in IMDM with 10% fetal bovine serum, plated at a density of 1,000 cells/ml in methylcellulose medium containing 4U/ml rhEpo, 10 ng/ml GM-CSF, 10 ng/ml rhIL-3, 50 ng/ml rhSCF, and scored by light and fluorescent microscopy 10 to 14 days after plating.
4. Transduced CD34⁺ cells were grown in liquid culture in three different conditions:
 - a) in the same medium used for transduction to maintain an undifferentiated phenotype;

- b) in X-VIVO-10 serum-free medium supplemented with 20 ng/ml rhSCF and 4U/ml rhEpo to induce erythroid differentiation;
 - c) in IMDM supplemented with 20% FBS, 20 ng/ml rhIL-3 and rhIL-6 to induce myeloid differentiation.
5. Cell phenotypes and transduction levels were determined by flow cytometry.

2.5.3.6 Infection by centrifugation (Spinoculation protocol).

1. Resuspend target cells in viral sup. +8µg/ml polybrene ($1.5-3 \times 10^5$ cells/ml)
2. Plate in 24 wells/plate (1ml) or 6 wells/plate (3ml).
3. Centrifuge at 1800 rpm 45 min at 30°C.
4. Harvest the cells, centrifuge in tube, resuspended in medium and incubate at 37°C 2h.
5. Repeat from step no. 1

2.5.4 Animal study methods.

All animals were maintained at the BL-2 level animal facility at DIBIT HSR, by the Charles Rivers staff appointed to manage it. NON-SCID mice were received from Charles Rivers, Italy.

2.5.4.1 Preparation of cells and injection of mice.

Cells were passaged as described. The experimental cells were washed twice in PBS, ensured to be a single cell suspension, and resuspended in 500 µl of PBS at numbers stated in the text. The cells were taken up into a 1 ml syringe with low gauge needle (26Gx 1/2") and transported to the mice.

Mice were prepared as described in the text. Tail vein injection was performed by holding a high intensity lamp to the tail of the mouse, and waiting for the veins to become clearly visible. The needle was then gently inserted into the visible vein and the plunger very slowly depressed to inject the cells into the mouse.

After successful completion of injection the mice were returned to their cages and maintained as described above. Gentilyn antibiotic was include in the water supply to prevent bacterial infections.

2.5.4.2 Termination of mice and extraction of the bone marrow.

Mice were terminated by carbon monoxide inhalation. The femurs and tibias were removed by surgical procedure, the bones cleaned of muscle and cartilage fibre, the

epiphyses cut and the femurs flushed with complete media. The bone marrow tissue was disaggregated by physical methods, the cells suspended in IMDM media containing 20% FCS, penicillin/streptomycin at 100 units per ml, and L-Glutamine (2mM).

Cells were returned into culture and assayed as described in the text.

2.5.4.3 Transplantation in NOD/SCID mice

A breeding colony of NOD/SCID mice (Jackson Laboratory, Bar Harbor, ME) was housed in a positive airflow ventilated rack, bred and maintained in micro isolators under specific pathogen-free condition. Mice to be transplanted were sub-lethally irradiated at 6 to 8 weeks of age with 350 cGy of total body irradiation from a Cesium source. Transduced human CD34⁺ cells (1.5 to 3×10^5) were administered within 24 hours after irradiation in a single intravenous (tail vein) injection. Mouse BM was analyzed by flow cytometry 5 to 11 weeks post-transplant. BM cells were plated at a density of 0.5 - 2×10^5 cells/ml in methylcellulose preparation preferentially supporting the growth of human colonies.

2.5.4.4 Transduction of murine bone marrow (BM), and BM transplantation

Murine BM cells were harvested from C57BL/6 mice (Charles River) by flushing femurs and tibiae, and infected for 24 hrs with viral stocks at a MOI of 200 in serum-free IMDM containing BIT serum substitute, and 50 ng/ml μ SCF, 10 ng/ml μ IL-3, 10 ng/ml μ Flt-3L, and 20 ng/ml μ IL-6. Transduced cells were injected (5×10^6 cells/mouse) into the tail vein of recipient 8-12-wk-old male C57BL/6-Ly-5.1 mice (B/6.SJL-CD45^a-Pep^{3b} from Jackson Laboratories), irradiated with two split doses of 400 cGy 4 hrs apart. Nine to eleven weeks after BMT, animals were sacrificed and hematopoietic tissues were collected for FACS analysis and clonogenic assay.

2.5.4.2 Flow cytometry analysis

Δ LNGFR expression in transduced cells was monitored by flow cytometry or epifluorescence microscopy using an indirect staining with the anti-human p75-NGFR monoclonal antibody (MoAb) 20.4 (ATCC) and the R-phycoerythrin (PE)-conjugated goat anti-mouse serum (Southern Biotechnology Associates, Birmingham, AL). Human cell surface phenotype and human cell engraftment in transplanted NOD/SCID mice were determined by flow cytometry using RPE-conjugated anti-human CD45 (DAKO, Glostrup, Denmark) to detect total human leukocytes, CD34 (Becton Dickinson, San

Jose, CA) for stem/progenitor cells, CD19 (DAKO) for B lymphocytes, CD14 (DAKO) for monocytes, CD13 (Caltag Laboratories, Burlingame, CA) for myeloid cells, GpA (DAKO) for erythrocytes. Indirect staining with biotinylated anti-p75-NGFR MoAb and Tricolor (TC)-conjugated Streptavidin (SAV Caltag Laboratories) was used to monitor transduction efficiency in each BM cell subpopulation. Mouse cell phenotyping was carried out using PE-conjugated anti-mouse TER-119 and Gr-1 (PharMingen) antibodies. The FITC-conjugated, anti-mouse CD45.2 MoAb (PharMingen) was used to evaluate donor-host chimerism in BM-transplanted mice. Isotype-matched non-specific antibodies were used as controls (Becton Dickinson). For cytofluorimetry analysis of non-erythroblastic cell populations, BM cells were lysed with 8.3% ammonium chloride to remove erythroid cells, and washed with PBS containing 0.1% bovine serum albumin and 0.01% sodium azide. Cells were then re-suspended at $1-2 \times 10^6$ cells/ml and incubated with mouse IgG (Sigma, Saint Louis, MS) and 5% human serum (HS), to block nonspecific binding to Fc receptor.

Chapters 3-8

RESULTS

Chapter 3

Generation of high titer lentiviral vectors for HSC gene transfer.

3.1 Viral supernatant production by co-transfection of 293T cells.

The development of gene therapy strategies to correct hemetopoietic and genetic disorders has been hampered by the low level of gene transfer into human HSCs using vectors derived from oncoretroviruses such as Moloney murin leukemia virus (MoMLV) virus (Kohn, 1997; Richter, 1997). Oncoretroviruses require cell division for integration and as repopulating HSCs are largely quiescent, oncoretroviral vectors are largely inefficient in these targets. Thus, much interest has recently been focused on vectors derived from lentiviruses, such as HIV-1, which have been shown to transduce a variety of non-dividing cells (Lewis and Emerman, 1994; Naldini et al., 1996). Furthermore, lentiviral (LV) vector viral particles pseudotyped with a glycoprotein G of VSV (VSV-G) envelope can enter a large variety of target cells as a result of the ubiquity of the VSV-G phospholipidic receptor, and can be easily concentrated by ultracentrifugation (Naldini et al., 1996).

To test the efficiency of HIV-1-derived, LV vectors as a gene transfer system into HSCs, we decided to produce a viral supernatant starting from a second-generation packaging system, kindly provided by Prof. Luigi Naldini (University of Turin). This system consists of three different plasmids (see Section 1.2.3) that are illustrated in Fig. 3.1. The multiple deleted pCMV.ΔR.8.91, which encodes Gag, Pol, Tat and Rev proteins, was used as a packaging construct (Fig. 3.1 A), while the pMD.G vector was used to express the heterologous VSV.G envelope (Fig. 3.1 B) (Zufferey et al., 1997). Finally, the pRRL.CMV.GFP.sin-18 (-18/C) vector, originally described by Dull *et al.*, was used as transfer vector (Dull et al., 1998). This HIV-1 vector contains a 400-nucleotide deletion in the U3 region of the 3' LTR (Fig. 3.1 C) (Zufferey et al., 1998). This deletion encompasses a region in which has been mapped all of the major determinants responsible for regulating HIV-1 LTR promoter activity, including the so-called negative responsive element (NRE), the two NFκB and the NF-ATc binding sites, the three SP1 binding sites, and also the TATA box (see Fig. 3.1 C). Since in the life cycle of all retroviruses, during the process of reverse transcription, the U3 region of the 3'LTR is duplicated to form the corresponding region of the 5'LTR (Coffin et al., 1997) this deletion will be transferred to the 5'LTR after infection of a target cell. Consequently, the transcriptional units from the LTRs in a provirus are eliminated. This

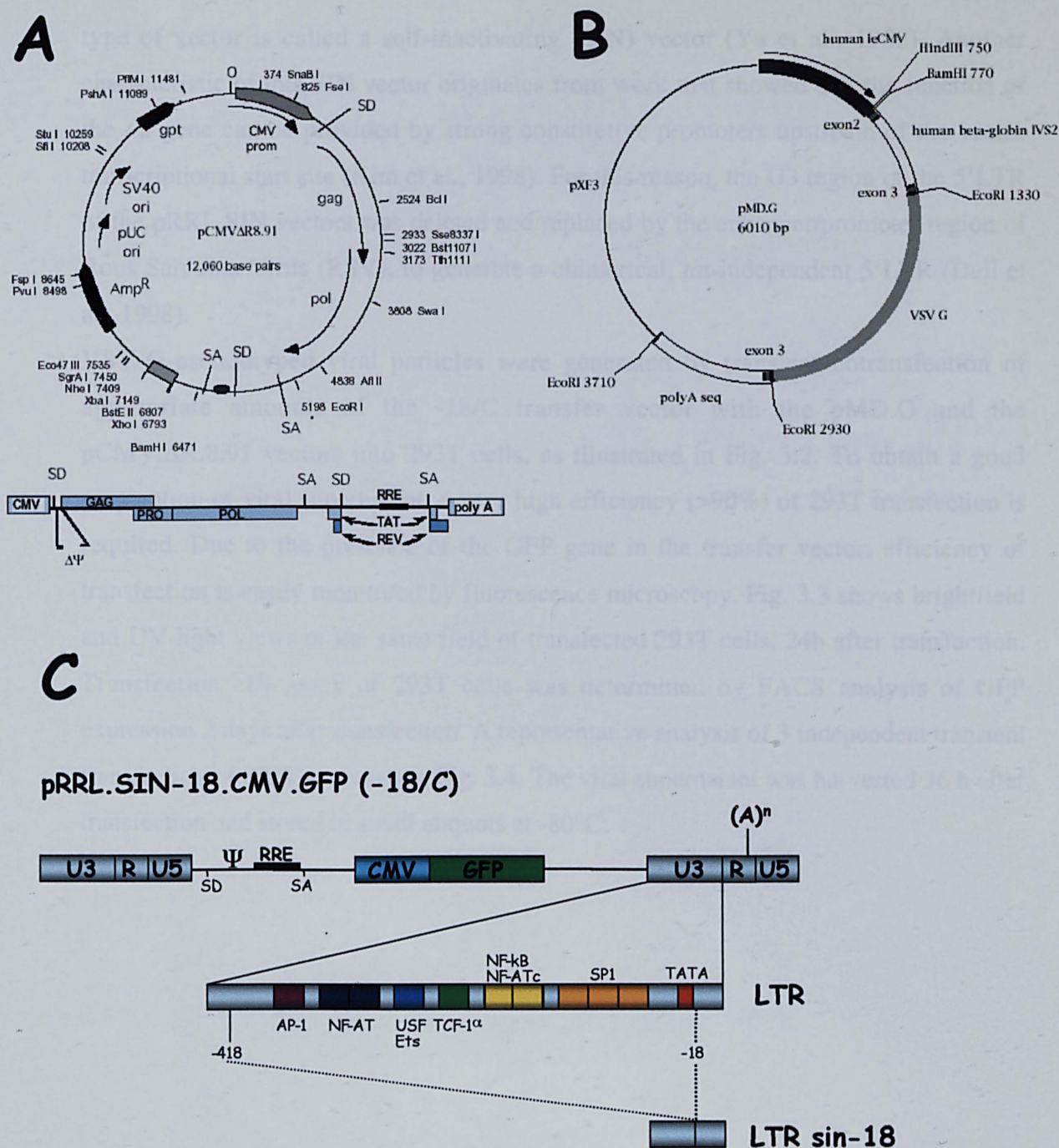


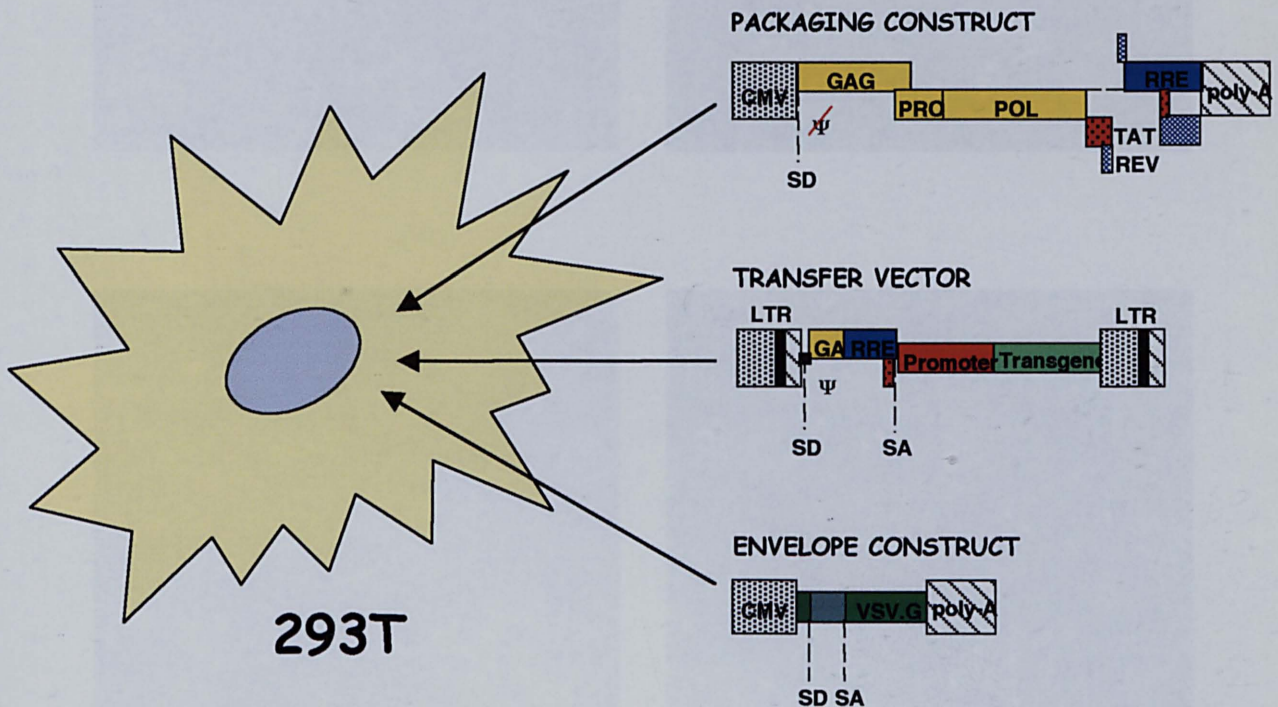
Figure 3.1. Schematic map of the three constructs used to generate second-generation LV particles. **(A)** pCMV.ΔR8.91 vector in its plasmid (upper) and proviral forms (bottom). This construct expresses Gag, Pol, Tat and Rev viral proteins under transcriptional control of the CMV promoter and is used as a packaging vector. **(B)** pMD.G vector in its plasmid form. The pMD.G vector, expressing the VSV.G protein under transcriptional control of the CMV promoter, is used as the envelope construct. **(C)** pRRL.sin-18.CMV.GFP (-18/C) vector in its proviral form. The 3' LTR carries a -418 to -18 deletion removing the U3 enhancer/promoter region of HIV-1. This vector contains GFP under transcriptional control of the CMV promoter and is used as a transfer vector. SD: splice donor; SA: splice acceptor; RRE: REV responsive element; (A)ⁿ: polyadenylation site; Ψ: packaging signal.

type of vector is called a self-inactivating (SIN) vector (Yu et al., 1986). Another characteristic of this SIN vector originates from work that showed that the function of the *tat* gene can be provided by strong constitutive promoters upstream of the vector transcriptional start site (Kim et al., 1998). For this reason, the U3 region of the 5'LTR of the pRRL.SIN vectors was deleted and replaced by the enhancer/promoter region of Rous Sarcoma Virus (RSV), to generate a chimerical, *tat*-independent 5'LTR (Dull et al., 1998).

VSV-G-pseudotyped viral particles were generated by transient cotransfection of appropriate amounts of the -18/C transfer vector with the pMD.G and the pCMV.ΔR.8.91 vectors into 293T cells, as illustrated in Fig. 3.2. To obtain a good preparation of viral supernatant, a very high efficiency (>90%) of 293T transfection is required. Due to the presence of the GFP gene in the transfer vector, efficiency of transfection is easily monitored by fluorescence microscopy. Fig. 3.3 shows brightfield and UV-light views of the same field of transfected 293T cells, 24h after transfection. Transfection efficiency of 293T cells was determined by FACS analysis of GFP expression 2 days after transfection. A representative analysis of 3 independent transient transfection of 293T is shown in Fig. 3.4. The viral supernatant was harvested 36 h after transfection and stored in small aliquots at -80°C.

SYSTEM TO MAKE LENTIVIRAL VECTORS

-Transient co-transfection of three constructs into 293T cells:



-collection of conditioning medium (c.m.) after 36 h

-End point titer on HeLa cells:

- transduction of 1×10^5 HeLa cells with serial dilution of c. m.
- FACS analysis after 48 h (transduction efficiency: TU/ml)
- TU/ml= n° of infected cells/dilution

Figure 3.2. Illustration of the different steps of lentiviral supernatant production.

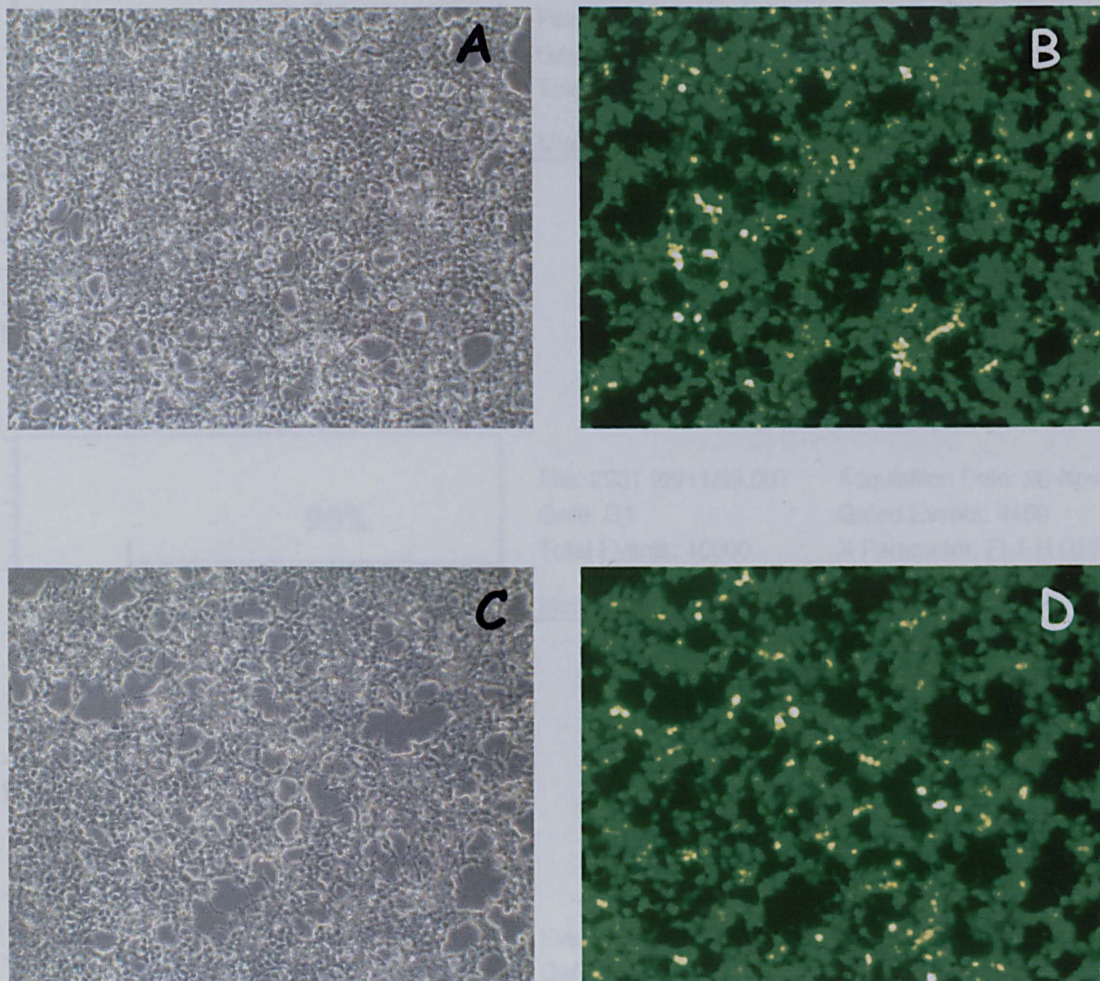
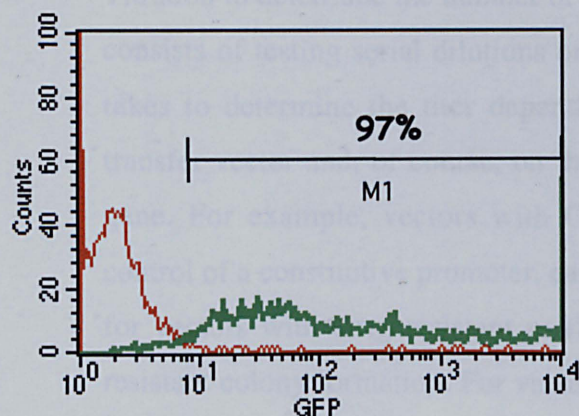
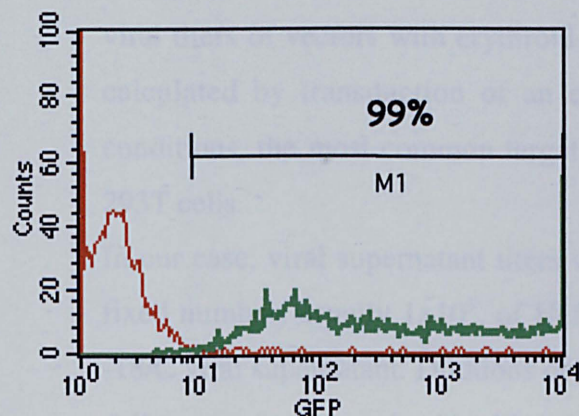


Figure 3.3. Expression of GFP in transiently transfected 293T cells. 293T cells were transfected with the three plasmids described in Fig. 3.1 and scored for GFP expression after 2 days by fluorescence microscopy. A,C, bright-field view of transfected 293T cells; B,D, green fluorescence view of the same fields (GFP-specific filters). Magnification: 10 X



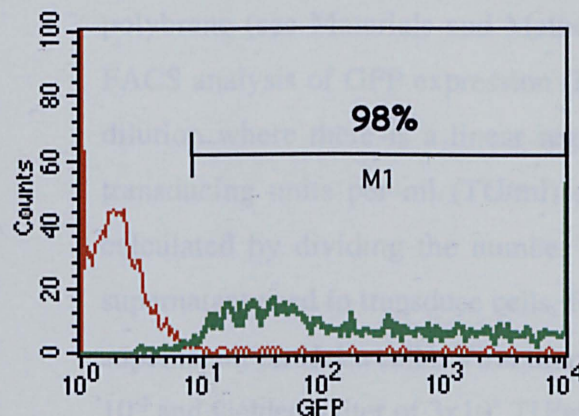
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 Gate: G1 Gated Events: 4186
 Total Events: 10000 X Parameter: FL1-H GFP (Log)

Marker	Left, Right	Events	% Gated	% Total	Mean
All	1, 9910	4186	100.00	41.86	1169.23
M1	8, 9910	4055	96.87	40.55	1206.83



File: 293T 28/11/99.007 Acquisition Date: 28-Nov-99
 Gate: G1 Gated Events: 4469
 Total Events: 10000 X Parameter: FL1-H GFP (Log)

Marker	Left, Right	Events	% Gated	% Total	Mean
All	1, 9910	4469	100.00	44.69	1912.20
M1	8, 9910	4459	99.78	44.59	1916.48



File: 293T 28/11/99.008 Acquisition Date: 28-Nov-99
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 Total Events: 10000 X Parameter: FL1-H GFP (Log)

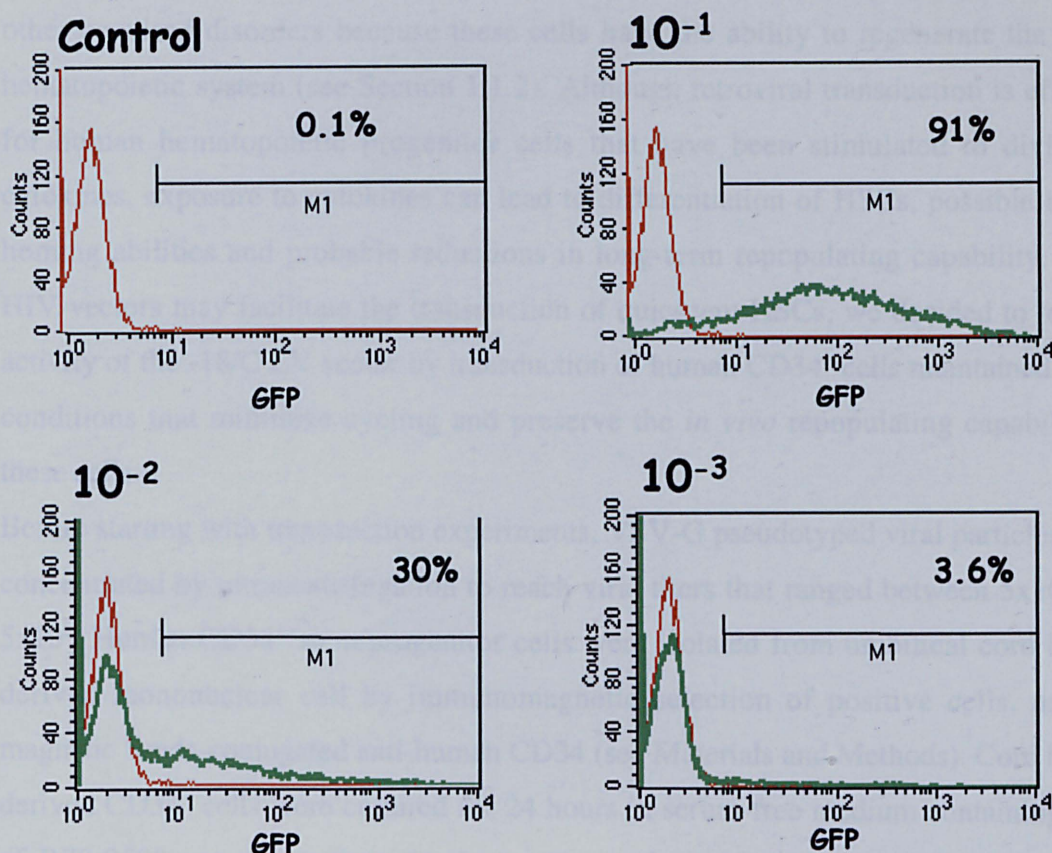
Marker	Left, Right	Events	% Gated	% Total	Mean
All	1, 9910	4322	100.00	43.22	1278.10
M1	8, 9910	4242	98.15	42.42	1302.08

Figure 3.4. FACS analysis of GFP expression in 3 different plates of transiently transfected 293T cells. 293T cells were transfected with the three plasmids described in Fig. 3.1 and monitored for GFP expression by single-channel cytofluorimetry (X axis). Expression profiles are indicated by a red (mock) or a green (-18/C vector) line respectively. Percentages of GFP expressing cells are indicated within each panel and by a red oval in the statistical analysis.

3.2 Viral supernatant titration.

Titration to determine the number of transducing units (TU)/ml in the viral vector stock consists of testing serial dilutions on appropriate target cells. The method and time it takes to determine the titer depends on the particular marker gene encoded in the transfer vector and, of course, on the type of transcriptional regulation of the marker gene. For example, vectors with GFP or luciferase markers, under transcriptional control of a constitutive promoter, can be harvested about 2-3 days post-infection, while for vectors with drug resistant markers it takes approximately two weeks for drug-resistant colony formation. For vectors in which the marker gene is regulated in a cell-lineage specific fashion, the use of the appropriate cell target is needed. For example, viral titers of vectors with erythroid-specific expression of the marker gene should be calculated by transduction of an erythroblastic cell line. However, under normal conditions, the most common target cells used to determine viral titers are HeLa and 293T cells.

In our case, viral supernatant titers were determined by transduction of HeLa cells. A fixed number, usually 1×10^5 , of HeLa cells were infected with serial dilutions of the -18/C viral supernatant. Dilutions of viral supernatant started from 10^{-1} up to 10^{-3} . HeLa cells were transduced with diluted viral supernatant in the presence of $8 \mu\text{g/ml}$ of polybrene (see Materials and Methods). Transduction efficiency was determined by FACS analysis of GFP expression (Fig. 3.5). Viral titers are usually calculated at that dilution where there is a linear regression of GFP expression and are expressed as transducing units per ml (TU/ml) of viral supernatant. This is a conventional unit calculated by dividing the number of GFP-expressing cells to the dilution of viral supernatant used to transduce cells. Fig. 3.5 shows a representative titration of the viral supernatant on HeLa cells. Viral titer of the -18/C vector was calculated at a dilution of 10^{-2} and yielded a titer of 3×10^6 TU/ml.



$$\text{TU/ml } (10^{-2}) = \frac{\text{no. cells} \times (\text{dilution})^{-1} \times \text{transd. efficiency}}{100}$$

$$\text{TU/ml } (10^{-2}) = 1 \times 10^5 \times 10^{-2} \times 0.3 = 3 \times 10^6$$

Figure 3.5. End-point titration of the -18/C vector viral supernatant in HeLa cells. HeLa cells transduced with serial dilutions of the viral supernatant (10^{-1} - 10^{-3}) were grown as bulk cultures and analyzed for GFP expression by single-channel cytofluorimetry (X axis). Expression profiles are indicated by a red (mock) or a green (-18/C vector) line respectively. Percentages of GFP expressing cells are indicated within each panel. Viral titer was calculated at a dilution of 10^{-2} as indicated in the bottom quadrant.

3.3 Transduction of CD34⁺ cells with lentiviral vectors.

Human HSCs are an attractive target for gene therapy of inherited disorders as well as other acquired disorders because these cells have the ability to regenerate the entire hematopoietic system (see Section 1.1.2). Although retroviral transduction is efficient for human hematopoietic progenitor cells that have been stimulated to divide by cytokines, exposure to cytokines can lead to differentiation of HSCs, possible loss of homing abilities and probable reductions in long-term repopulating capability. Since HIV vectors may facilitate the transduction of quiescent HSCs, we decided to test the activity of the -18/C LV vector by transduction of human CD34⁺ cells maintained under conditions that minimize cycling and preserve the *in vivo* repopulating capability of these cells.

Before starting with transduction experiments, VSV-G pseudotyped viral particles were concentrated by ultracentrifugation to reach viral titers that ranged between 5×10^8 and 5×10^9 . Human CD34⁺ stem/progenitor cells were isolated from umbilical cord blood-derived mononuclear cell by immunomagnetic selection of positive cells, using a magnetic beads-conjugated anti-human CD34 (see Materials and Methods). Cord blood-derived CD34⁺ cells were cultured for 24 hours in serum-free medium containing 20% of BIT 9500 serum-substitute in the presence of cytokines and transduced with the pRRL.CMV.GFP.sin-18 vector at a multiplicity of infection (MOI) of 100, determined on HeLa cells. This means that, theoretically, 100 TU were used to infect one CD34⁺ cell. After three days post infection, cells were analyzed by flow cytometry for CD34, CD38 and GFP expression (Fig. 3.6). Under these conditions, more than 70% of the cells retained a CD34⁺ phenotype (Fig. 3.6, upper panel), while about 50% of CD34⁺ cells were also CD38⁻, the more immature phenotype of the human HSCs (Fig. 3.6, middle panel). GFP expression was evaluated in the CD38⁻ and CD38⁺ subpopulation of CD34⁺ cells (Fig. 3.6, bottom panels). More than 54% of total CD34⁺ cells were transduced as measured by the expression of GFP driven by the internal CMV promoter.

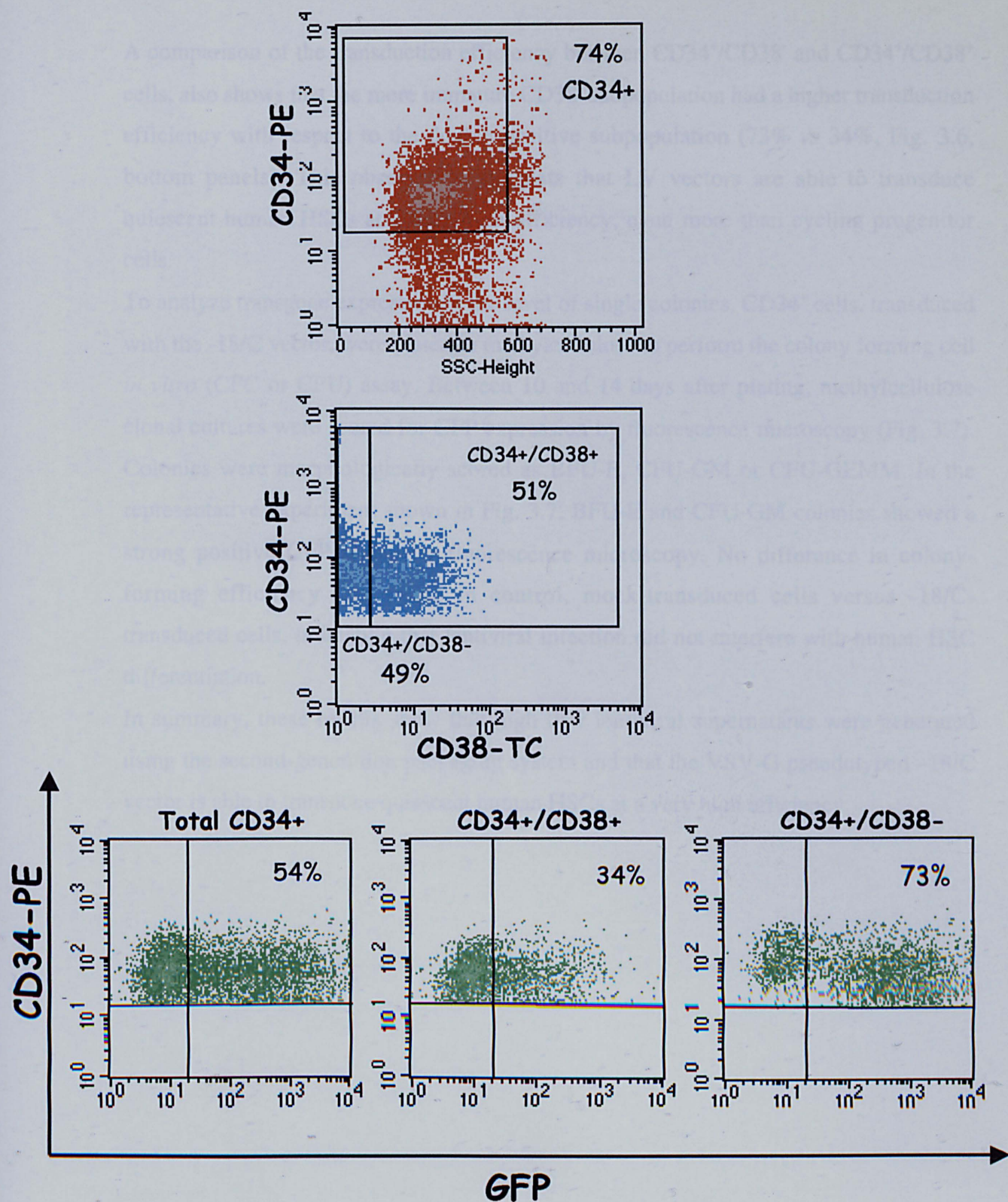


Figure 3.6. Expression of CD34, CD38 (upper panels) and GFP (lower panels) in liquid culture of human CD34⁺ hematopoietic stem/progenitor cells transduced with the -18/C vector and analyzed 4 days after infection. Cells were analyzed by triple-immunofluorescence flow cytometry after staining with a TC-conjugated anti-CD38 antibody and PE-conjugated anti-CD34 antibody. % values are indicated in the gated areas (upper and middle panels). % values in each quadrant indicate cells double positive for CD34 and GFP expression (lower panels).

A comparison of the transduction efficiency between CD34⁺/CD38⁻ and CD34⁺/CD38⁺ cells, also shows that the more immature CD38⁻ subpopulation had a higher transduction efficiency with respect to the double positive subpopulation (73% vs 34%, Fig. 3.6, bottom panels). This observation suggests that LV vectors are able to transduce quiescent human HSCs at a very high efficiency, even more than cycling progenitor cells.

To analyze transgene expression at the level of single colonies, CD34⁺ cells, transduced with the -18/C vector, were plated in methylcellulose to perform the colony forming cell *in vitro* (CFC or CFU) assay. Between 10 and 14 days after plating, methylcellulose clonal cultures were scored for GFP expression by fluorescence microscopy (Fig. 3.7). Colonies were morphologically scored as BFU-E, CFU-GM or CFU-GEMM. In the representative experiment shown in Fig. 3.7, BFU-E and CFU-GM colonies showed a strong positive GFP signal by fluorescence microscopy. No difference in colony-forming efficiency was found in control, mock-transduced cells versus -18/C-transduced cells, indicating that lentiviral infection did not interfere with human HSC differentiation.

In summary, these results show that high titer lentiviral supernatants were generated using the second-generation packaging system and that the VSV-G pseudotyped -18/C vector is able to transduce quiescent human HSCs at a very high efficiency.

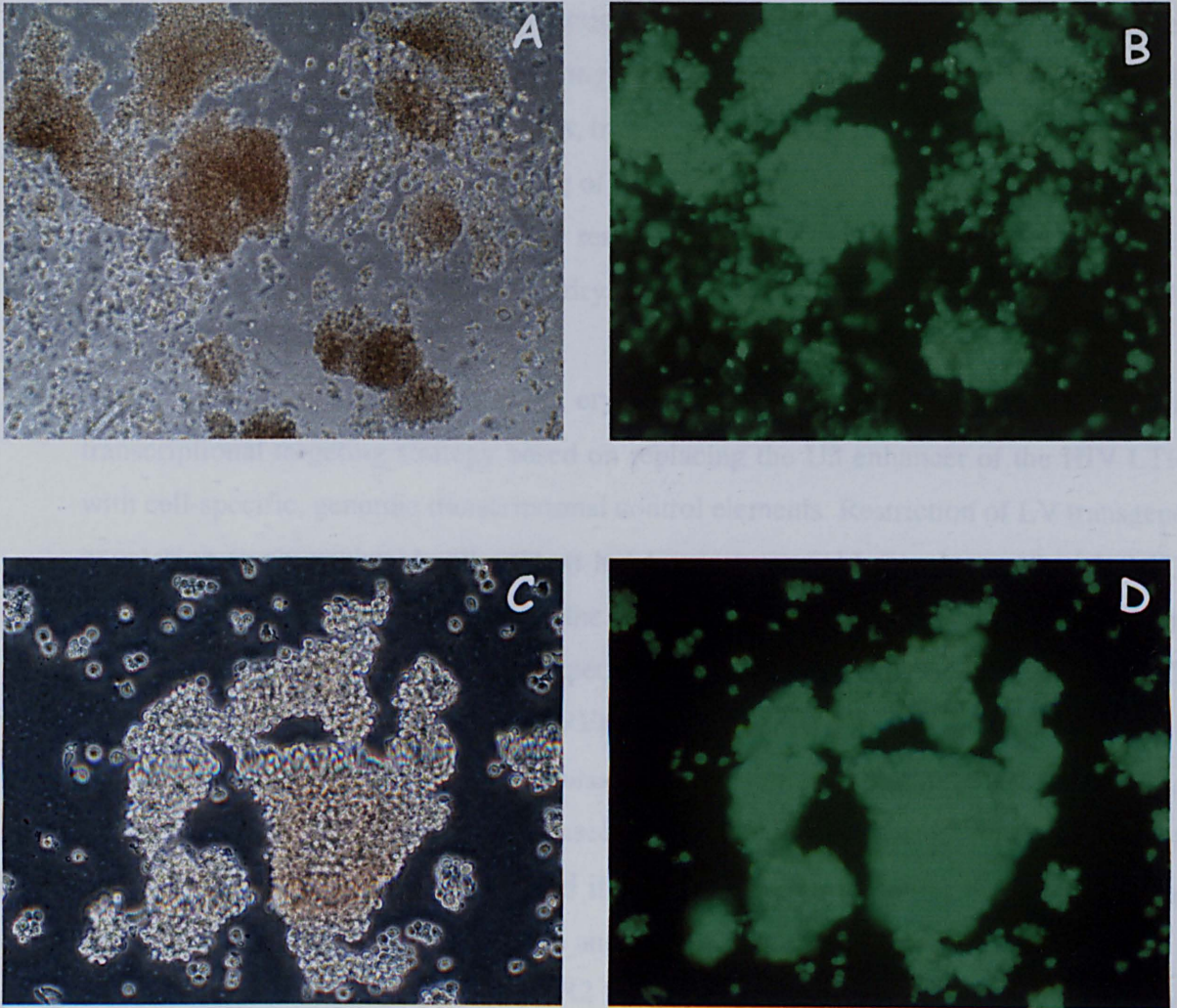


Figure 3.7. Expression of GFP in methylcellulose colonies derived from CD34⁺ human hematopoietic progenitors transduced with the -18/C lentiviral vector (see Fig. 1.3 C). A,C, bright-field view of fully mature BFU-E colonies 14 days after plating; B,D, green fluorescence view of the same fields (GFP-specific filters). Magnification: 10 X

Chapter 4

Development of erythroid specific Lentiviral vectors .

4.1 Transcriptional targeting of Lentiviral vectors by LTR enhancer replacement.

Correction of genetic disorders affecting specific progeny of HSCs (for example, hemoglobinopathies) requires restricting expression of the therapeutic gene in a cell lineage-specific fashion. In these cases, transcriptional targeting of the transferred gene is mandatory. Transcriptional targeting of LV vectors has been attempted by placing the gene of interest under the control of restricted internal promoters (Cui et al., 2002; Marodon et al., 2003; Moreau-Gaudry et al., 2001) in the framework of a self-inactivating (SIN) vector.

In order to obtain a high-expressing, erythroid-specific LV vectors, we developed a transcriptional targeting strategy based on replacing the U3 enhancer of the HIV LTR with cell-specific, genomic transcriptional control elements. Restriction of LV transgene expression to an erythroid cell context has been attempted by replacing the viral U3 enhancer elements in the 3'LTR with the DNase hypersensitive site region (HS2) from upstream of the GATA-1 erythroid-specific promoter (-856 to -655). GATA-1 HS2 contains an autoregulatory element (ARE) able to restrict transcription of a heterologous promoter to human or murine erythroblastic cell lines (Nicolis et al., 1991; Tsai et al., 1991). This same element has been used recently in our laboratory in the context a MoMLV-derived retroviral vector and it has been shown to confer erythroid lineage specificity upon transduction of human and murine HSCs (Grande et al., 1999).

The 3' LTR of the first generation pHR2 LV vector (Dull et al., 1998) has been used to generate self-inactivating LV vectors (SIN) carrying an almost complete deletion of the U3 region, from position -418 to -40 with respect to the transcription start site (Fig. 4.1). The same deletion in the U3 region has also been replaced by a 200-bp human genomic fragment containing the autoregulatory element of GATA-1 (ARE or HS2) described before (Fig 4.1). The -40 deleted LTR and the GATA-1/HIV-1 chimeric LTR (GATA-LTR), were used to replace the 3' LTR of the pRRL.SIN lentiviral vector, carrying GFP as a reporter gene (Zufferey et al., 1998). The resulting vectors, pRRL.sin-40.GFP (-40 in Fig. 4.1) and pRRL.GATA.GFP (G in Fig. 4.1), were used as transfer vectors and were pseudotyped with the VSV-G envelope by the second-generation of lentiviral packaging system described in Section 3.1.

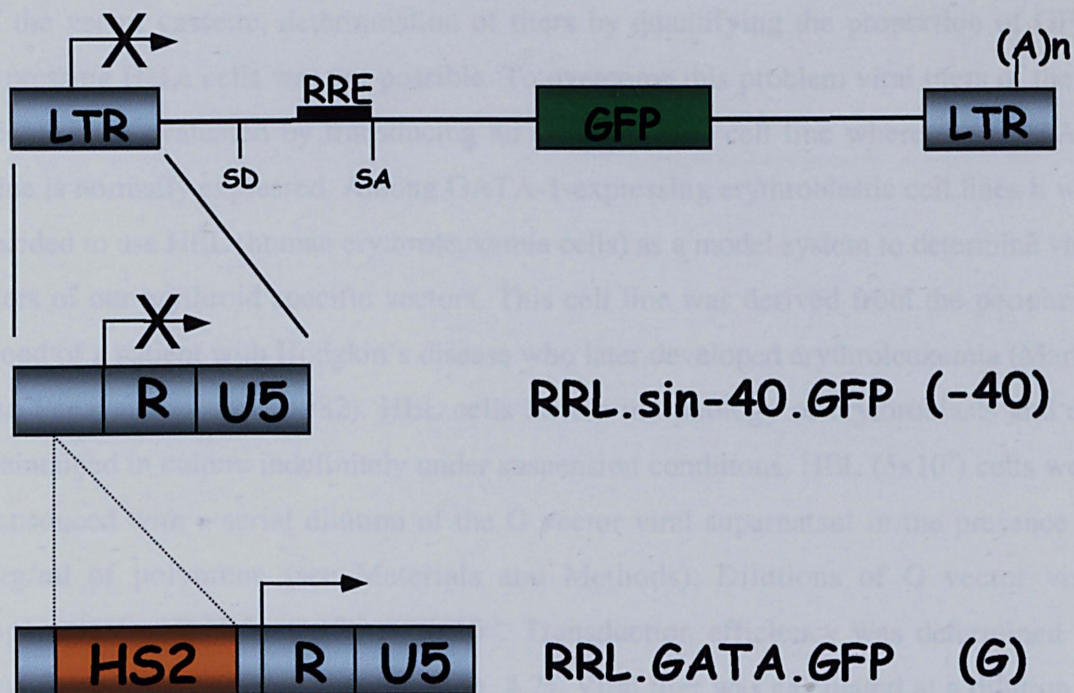
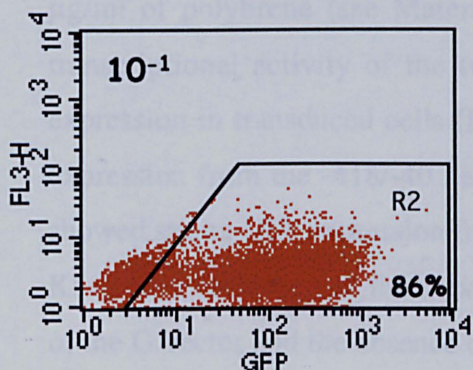
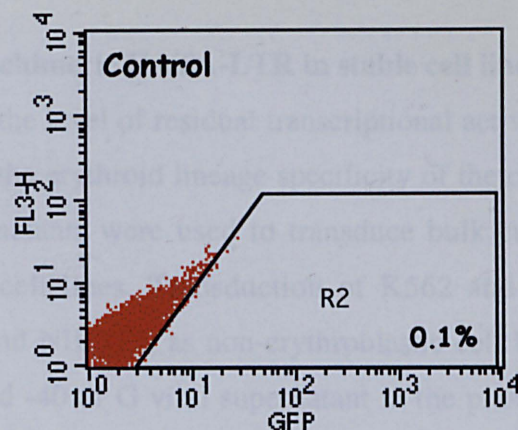
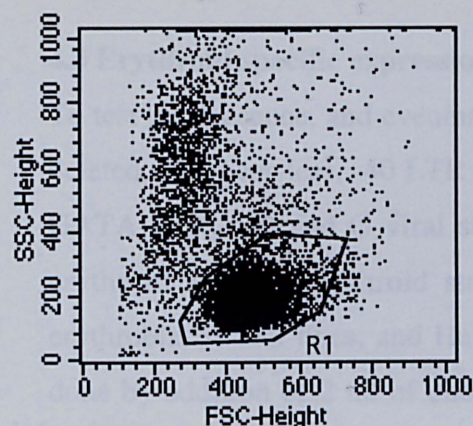


Figure 4.1. Schematic map of the RRL.sin-40.GFP (-40) and the RRL.GATA.GFP (G) LV vectors. In the -40 vector, the LTR carries a -418 to -40 deletion removing the U3 enhancer, while in the G vector the same region is replaced by the GATA-1 HS2 auto regulatory enhancer. An arrow indicates the transcription start site of the chimeric LTR promoter. A crossed arrow indicates the disabled LTR promoter in the -40 vector. SD: splice donor; SA: splice acceptor; RRE: REV responsive element; (A)ⁿ: polyadenylation site.

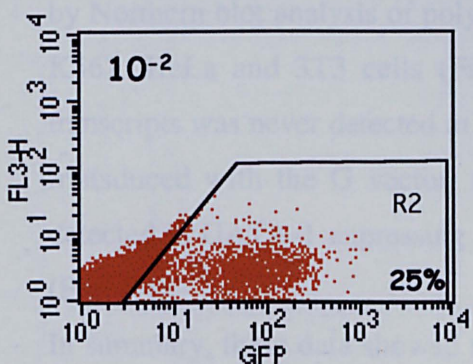
4.2 Viral titration of GATA-LTR vectors.

Viral titers are usually determined by transducing HeLa cells with serial dilutions of viral supernatant (see section). But, in this case, due to the expected erythroid specificity of the vector cassette, determination of titers by quantifying the proportion of GFP-expressing HeLa cells was not possible. To overcome this problem viral titers of the G vector were evaluated by transducing an erythroblastic cell line where the GATA-1 gene is normally expressed. Among GATA-1-expressing erythroblastic cell lines it was decided to use HEL (human erythroleukemia cells) as a model system to determine viral titers of our erythroid specific vectors. This cell line was derived from the peripheral blood of a patient with Hodgkin's disease who later developed erythroleukemia (Martin and Papayannopoulou, 1982). HEL cells have a morphology of erythroblasts and are maintained in culture indefinitely under suspension conditions. HEL (5×10^5) cells were transduced with a serial dilution of the G vector viral supernatant in the presence of $4 \mu\text{g/ml}$ of polybrene (see Materials and Methods). Dilutions of G vector viral supernatant started from 10^{-1} up to 10^{-3} . Transduction efficiency was determined by FACS analysis of GFP expression (Fig. 4.2). Viral titer was calculated at a dilution of 10^{-3} and yielded a titer of 1.5×10^7 TU/ml. Usually, vector titers ranged from 2×10^6 to 5×10^7 TU/ml, with a mean of 4.5×10^6 .



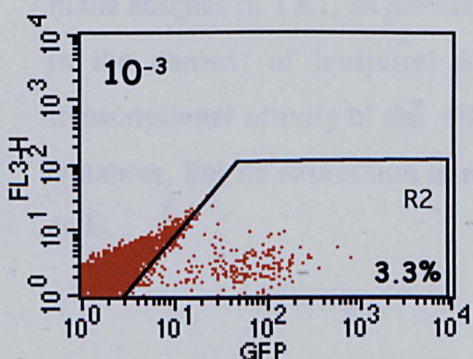
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Gated Events: 7292 Total Events: 10000
X Parameter: FL1-H GFP (Log) Y Parameter: FL3-H (Log)

Region	Events	% Gated	% Total	X Mean
R1	7292	100.00	72.92	182.96
R2	6315	86.60	63.15	210.70



File: 24/1/01.013 Gate: G1
Gated Events: 7612 Total Events: 10000
X Parameter: FL1-H GFP (Log) Y Parameter: FL3-H (Log)

Region	Events	% Gated	% Total	X Mean
R1	7612	100.00	76.12	23.01
R2	1893	24.87	18.93	84.03



File: 24/1/01.014 Gate: G1
Gated Events: 7708 Total Events: 10000
X Parameter: FL1-H GFP (Log) Y Parameter: FL3-H (Log)

Region	Events	% Gated	% Total	X Mean
R1	7708	100.00	77.08	4.77
R2	256	3.32	2.56	66.02

$$\text{TU/ml (10}^{-3}\text{)} = 5 \times 10^5 \times 10^{-3} \times 0.03 = 1.5 \times 10^7$$

Figure 4.2. End-point titration of G vector viral supernatant on HEL cells. HEL cells transduced with serial dilutions of the viral supernatant (10^{-1} - 10^{-3}) were grown as bulk cultures and analysed for GFP expression by single-channel cytofluorimetry (X axis). Percentages of GFP expressing cells are indicated in the gated area and by a red oval in the statistic panel. Viral titer was calculated at a dilution of 10^{-2} as indicated.

4.3 Erythroid specific expression of chimeric GATA-LTR in stable cell lines.

To test the presence, and eventually, the level of residual transcriptional activity of the deleted, enhancer-less, -40 LTR and the erythroid lineage specificity of the chimerical GATA-LTR, -40 and G viral supernatants were used to transduce bulk cultures of erythroid and non-erythroid stable cell lines. Transduction of K562 and HEL, as erythroblastic cell lines, and HeLa and NIH3T3, as non-erythroblastic cell lines, was done by addition of 2 ml of undiluted -40 or G viral supernatant in the presence of 4 µg/ml of polybrene (see Materials and Methods). Five days after transduction, the transcriptional activity of the two vectors was assessed by FACS analysis of GFP expression in transduced cells (Fig 4.3). As expected, the -40 vector showed no GFP expression from the -418/-40 deleted LTR (thin lines in Fig. 4.3), while the G vector showed strong GFP expression from the chimeric GATA-LTR, but only in the HEL and K562 erythroblastic cell lines (bold lines in Fig. 4.3). The erythroid specific expression of the G vector and the absence of transcriptional activity in -40 vector were confirmed by Northern blot analysis of poly-A⁺ RNA extracted from G- and -40-transduced HEL, K562, HeLa and 3T3 cells (Fig. 4.4). As expected, accumulation of LTR-driven transcripts was never detected in cells transduced with the -40 vector (Fig. 4.4). In cells transduced with the G vector, transcripts derived from the GATA-LTR were only detected in GATA-1-expressing HEL and K562 cells and not in U937 and HeLa cells (Fig. 4.4).

In summary, these data showed that the enhancer-less HIV-1 LTR is virtually inactive in the absence of TAT, as previously reported for two similar U3 deletions (-45 and -36) in the context of lentiviral SIN vectors (Zufferey et al., 1998). However, the transcriptional activity of the -40 LTR is restored by the insertion of the GATA-1 HS2 enhancer, but its expression is restricted only to GATA-1-expressing K562 and HEL cells.

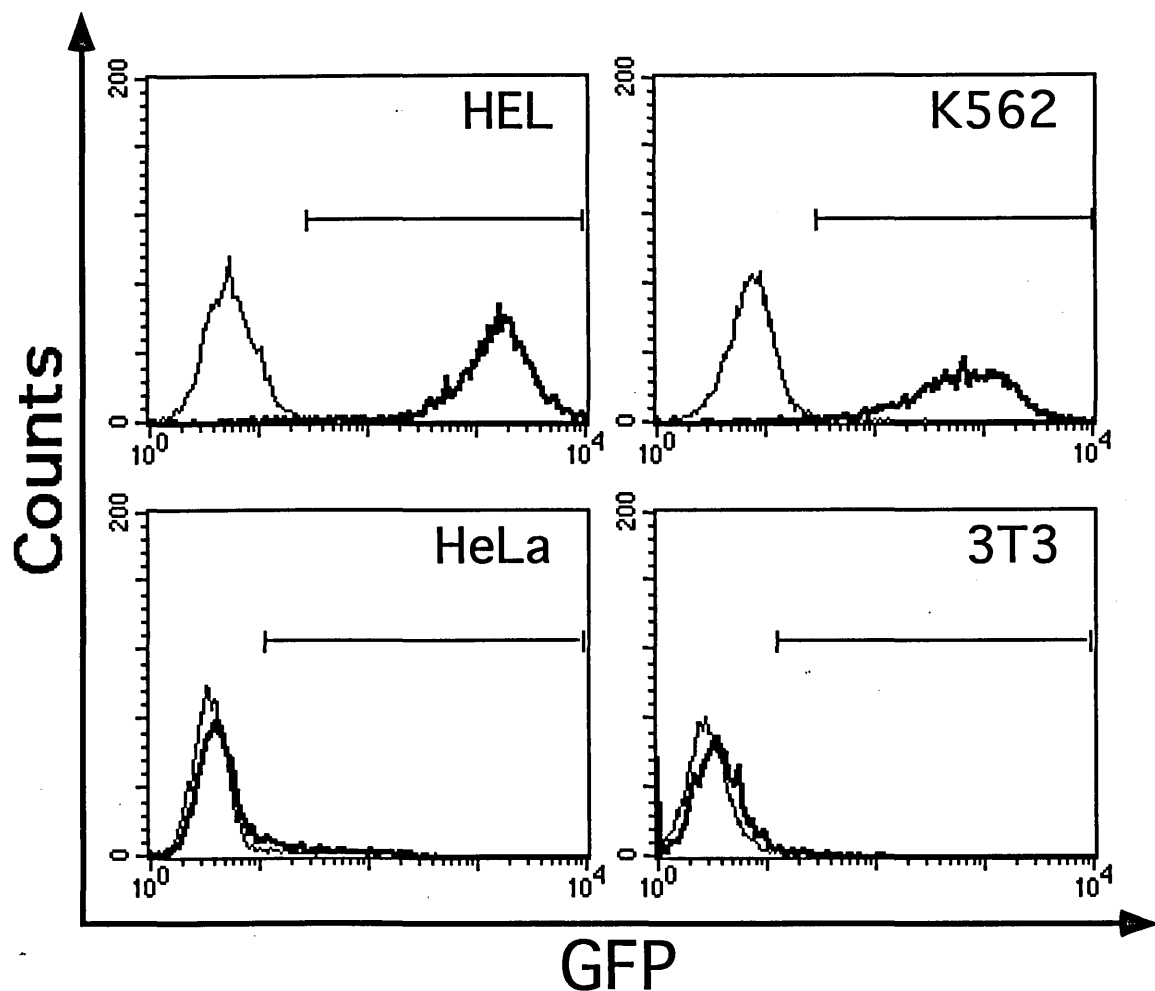


Figure 4.3. Analysis of GFP expression in cell lines transduced with the G LV vector, carrying a GFP gene under the control of the GATA-1 HS2-modified LTR (see Fig. 1A), and with a control -40 vector, carrying a GFP gene under the control of an enhancer-less LTR. Human erythroblastic HEL and K562 and non-hematopoietic HeLa, and murine fibroblast NIH-3T3 cells, were infected with the two vectors at an MOI of 10, grown as bulk cultures, and analyzed for GFP expression by single-channel cytofluorimetry (X axis). Expression profiles are indicated by a thin (-40 vector) or a bold (G vector) line respectively.

4.3 Genomic stability of LTR-modified lentiviral vectors

Several reports have shown that LTR modification, especially in oncogenic vectors, caused frequent rearrangements of the vector genome (Kaptein et al., 1998). To determine whether a similar event occurs in the context of an HIV-1 backbone, we decided to perform genome Southern blot analysis of HFL cells transduced with G and -40 vectors. Genomes were extracted 4 weeks after transduction, was

extracted with AflIII and digested with AflIII. The resulting fragments were separated on a 1% agarose gel and transferred to a nylon membrane. The membrane was probed with a GFP probe. The results are shown in Figure 4.5 A. Southern blot analysis showed that transduced HFL cells contained the expected GFP fragment (1.3 kb) and with the expected proviral size (4.4 kb). The results are shown in Figure 4.5 A. Southern blot analysis showed that transduced HFL cells contained the expected GFP fragment (1.3 kb) and with the expected proviral size (4.4 kb).

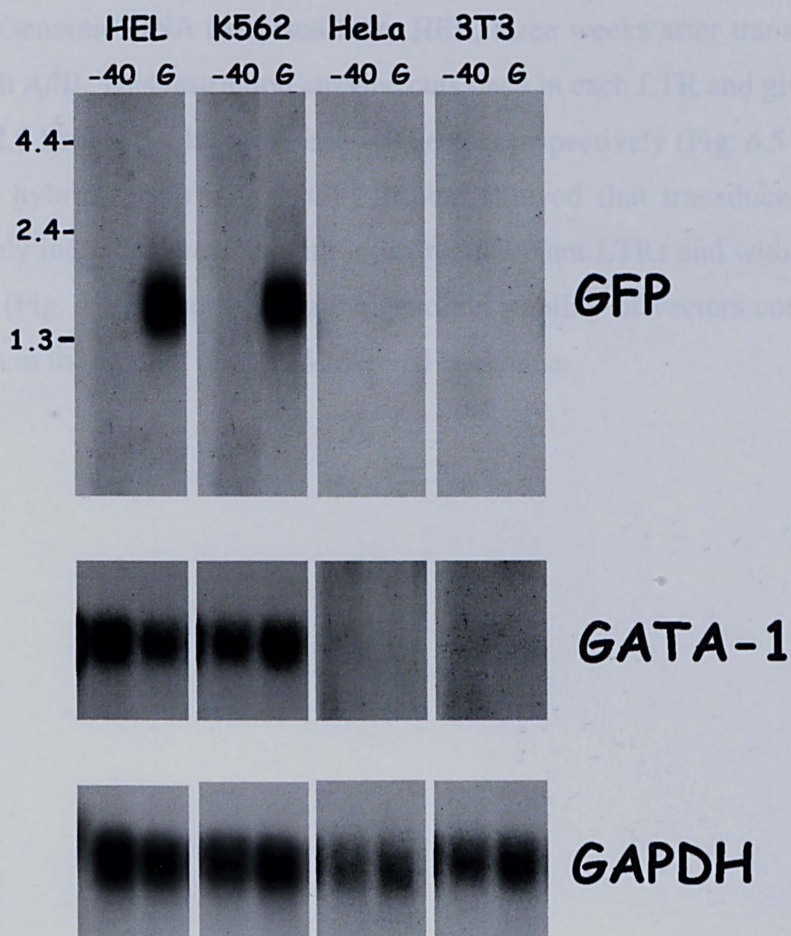


Figure 4.4. Northern blot analysis of poly-A⁺ RNA extracted from HEL, K562, HeLa and NIH-3T3 cell lines transduced with the G or the -40 LV vectors and hybridized with GFP (upper panels), GATA-1 (middle panels) and GAPDH (lower panels) probes. Molecular weight markers (in kilobases) are indicated on the left.

4.3 Genomic stability of LTR-modified lentiviral vectors.

Several reports have shown that LTR modification, especially in oncoretroviral vectors, caused frequent rearrangements of the vector genome (Kaptein et al., 1998). To determine whether a similar event occurs in the context of an HIV-1 backbone, we decided to perform genomic Southern blot analysis of HEL cells transduced with G and -40 vectors. Genomic DNA extracted from HEL, three weeks after transduction, was restricted with *Afl*III. This restriction enzyme cuts once in each LTR and gives a proviral fragment of 2.6 kb and 2.4 kb for G and -40 vectors respectively (Fig. 4.5 A). Southern blotting and hybridization with a GFP probe, showed that transduced HEL cells harbored stably integrated vectors with intact recombinant LTRs and with the expected proviral size (Fig. 4.5 B), confirming the genomic stability of vectors containing LTR modifications in the context of an HIV-derived backbone.

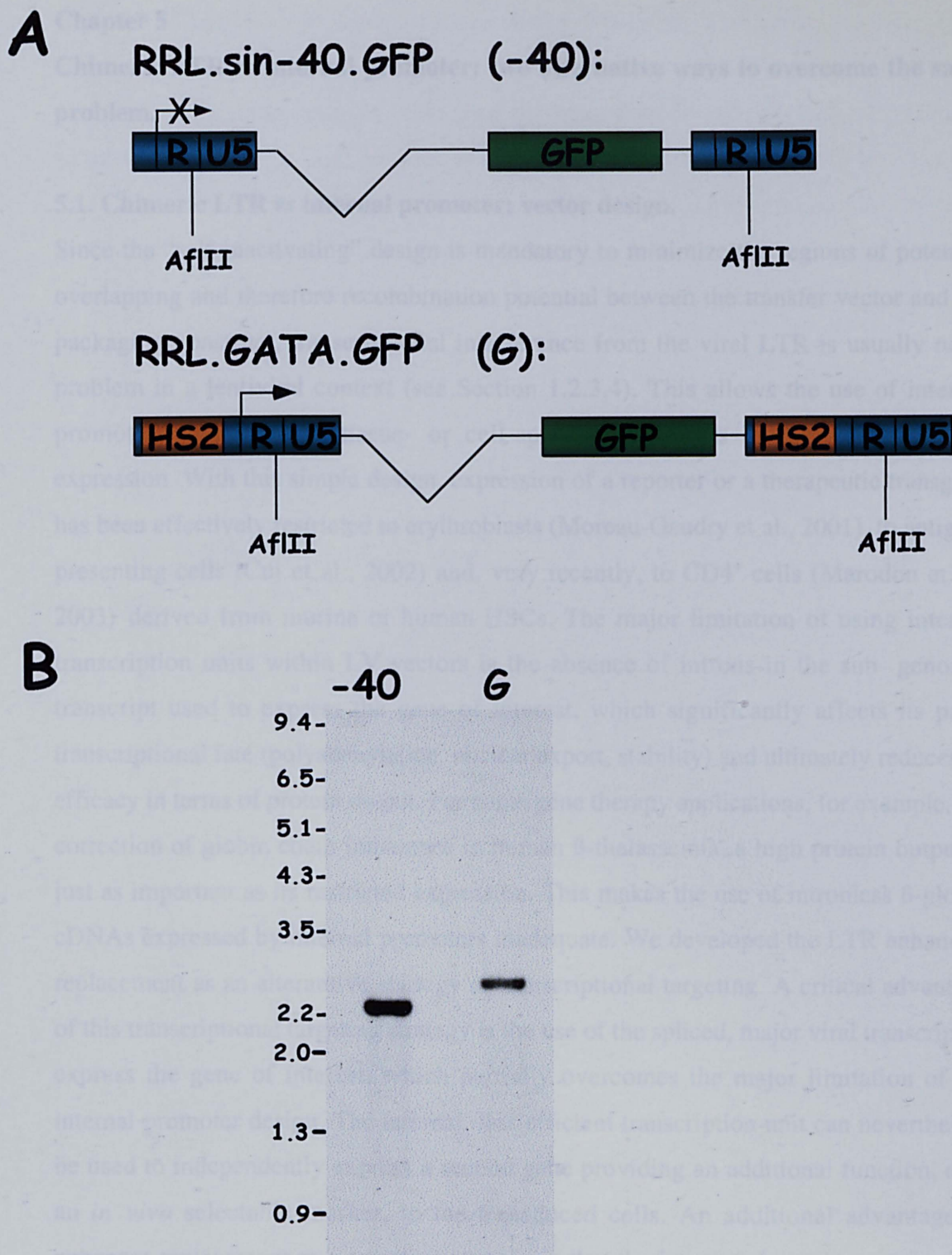


Figure 4.5. (A) Schematic map of the RRL.sin-40.GFP (-40) and the RRL.GATA.GFP (G) LV vectors in their proviral form, previously described in Fig. 3.1. Restriction sites used for the Southern blot analysis are indicated. (B) Southern blot analysis of genomic DNA extracted from HEL cell lines transduced with the -40 or the G vectors, digested with *Af*III, and hybridized to a GFP probe. Molecular weight markers (in kilobases) are indicated on the left.

Chapter 5

Chimeric LTR vs internal promoter: two alternative ways to overcome the same problem.

5.1. Chimeric LTR vs internal promoter: vector design.

Since the “self-inactivating” design is mandatory to minimize the regions of potential overlapping and therefore recombination potential between the transfer vector and the packaging construct, transcriptional interference from the viral LTR is usually not a problem in a lentiviral context (see Section 1.2.3.4). This allows the use of internal promoters combined to tissue- or cell-specific enhancers to regulate transgene expression. With this simple design, expression of a reporter or a therapeutic transgene has been effectively restricted to erythroblasts (Moreau-Gaudry et al., 2001), to antigen-presenting cells (Cui et al., 2002) and, very recently, to CD4⁺ cells (Marodon et al., 2003) derived from murine or human HSCs. The major limitation of using internal transcription units within LV vectors is the absence of introns in the sub-genomic transcript used to express the gene of interest, which significantly affects its post-transcriptional fate (polyadenylation, nuclear export, stability) and ultimately reduces its efficacy in terms of protein output. For some gene therapy applications, for example, the correction of globin chain imbalance in human β -thalassemia, a high protein output is just as important as its restricted expression. This makes the use of intronless β -globin cDNAs expressed by internal promoters inadequate. We developed the LTR enhancer-replacement as an alternative strategy of transcriptional targeting. A critical advantage of this transcriptional targeting strategy is the use of the spliced, major viral transcript to express the gene of interest, which partially overcomes the major limitation of the internal promoter design. The internal, less efficient transcription unit can nevertheless be used to independently express a second gene providing an additional function, e.g., an *in vivo* selectable marker, to the transduced cells. An additional advantage of enhancer replacement as a targeting strategy is that the integrated provirus carries two copies of a genomic enhancer within the two LTRs. The presence of two active enhancers flanking the transgene transcription unit could reduce the chances of chromatin-mediated inactivation of transcription, which is known to affect the long-term maintenance of retroviral transgene expression *in vivo*, particularly in stem cells (Challita and Kohn, 1994).

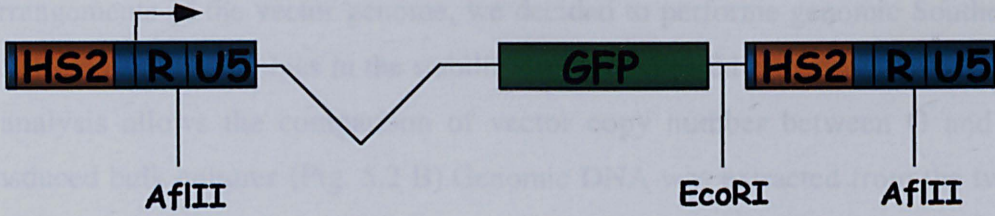
In order to test the advantages of our vector design as a transcriptional targeting approach, we compared the activity of the G vector with that of a RRL.sin-18.GATA.GFP (-18/G) vector containing the same combination of enhancer/promoter in the conventional internal position in the context of a SIN LV vector (Fig. 5.1 A). In these two vectors the GFP is under control of the same transcriptional unit, the only difference is that in the G vector transcription starts from the LTR, while in the -18/G vector transcription starts from the internal position, downstream of the splice donor and acceptor sites (Fig. 5.1 A).

5.2 Chimeric LTR vs internal promoter: transgene expression efficiency

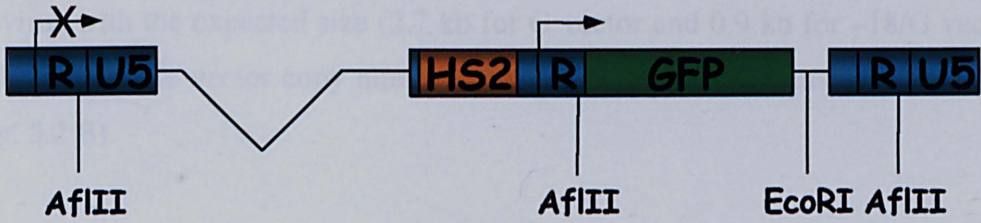
In the first instance, we decided to test the performance of the G and -18/G vectors under the best conditions of transduction in order to compare populations of cells that are fully infected. For this reason, HEL cells were transduced with the G and -18/G vectors at a relatively high MOI (around 10), using the spinoculation protocol described in Materials and Methods. To study the stability of GFP expression over time, the transduced HEL cells were maintained in culture as bulk populations for two months and were monitored for GFP expression by FACS analysis every two weeks. Transduction efficiency was determined five days after transduction by FACS analysis of GFP expression and it was very similar for both vectors (virtually 100%, Fig. 5.1 B). However, when we compare the mean fluorescence intensity (MFI) of GFP expression of both vectors, a four-fold higher GFP expression is present in cells transduced with the G vector with respect to those transduced with the -18/G vector (MFI: 2300 vs 560, Fig. 5.1 B). GFP expression of the integrated proviruses in the two bulk populations monitored by FACS analysis showed no substantial difference in terms of long-term stability of transgene expression between the G and -18/G vectors.

The higher efficiency in transgene expression of the GATA-LTR was confirmed at a molecular level by Northern blot analysis. The Poly-A⁺ mRNAs were isolated from G and -18/G transduced HEL bulk populations of cells four weeks after transduction and were hybridized to a GFP or a GAPDH probes (Fig. 5.2 A). Northern blot analysis showed that genomic transcripts from the G vector were accumulated in HEL cells at a level five-fold higher than that of the sub-genomic transcript from the -18/G vector, as quantified by phosphorimaging after normalization for GAPDH mRNA content (Fig. 5.2 A).

A RRL.GATA.GFP (G):



RRL.sin-18.GATA.GFP (-18/G):



B

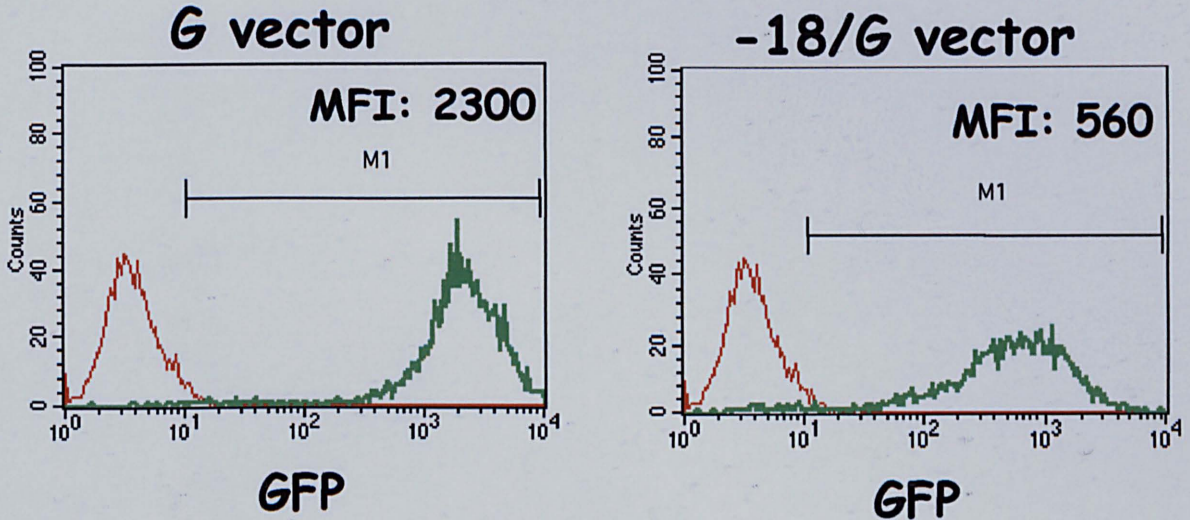


Figure 5.1. (A) Schematic map of the RRL.GATA.GFP (G) and the RRL.sin-18.GATA.GFP (-18/G) vectors in their proviral form. HS2 indicates the GATA-1 HS2 auto regulatory enhancer replacing the HIV LTR U3 region in the G vector. An arrow indicates the transcription start site of the chimeric LTR promoter. A crossed arrow indicates the disabled LTR promoter in the -18/G vector. Restriction sites used for the Southern blot analysis shown in Fig. 3.4 B are indicated. (B) Analysis of GFP expression in HEL cells transduced with the -18/G and G LV vector. HEL cells were infected with the two vectors at an MOI of 10, grown as bulk cultures and analyzed for GFP expression by single-channel cytofluorimetry (X axis). Expression profiles are indicated by a red (mock infected cells) or a green (infected cells) peaks respectively. Mean fluorescence intensity (MFI) of GFP expression is in arbitrary units.

5.3 Chimeric LTR vs internal promoter: vector DNA stability

Since the presence of repeated sequences in the vector DNA enhances the chances of rearrangements of the vector genome, we decided to performe genomic Southern blot analysis to exclude any bias in the stability of the integrated proviruses. The same type of analysis allows the comparison of vector copy number between G and -18/G transduced bulk cultures (Fig. 5.2 B). Genomic DNA was extracted from the two bulk populations of cells and restricted with *Afl*II. This restriction enzyme cuts in the R region and, for this reason, cuts twice in the G and three times in the -18/G vector (Fig. 5.1 A). Hybridization with a GFP probe showed that both vectors transmitted an intact provirus with the expected size (2.7 kb for G vector and 0.9 kb for -18/G vector) and with comparable vector copy number between G and -18/G transduced bulk cultures (Fig. 5.2 B).

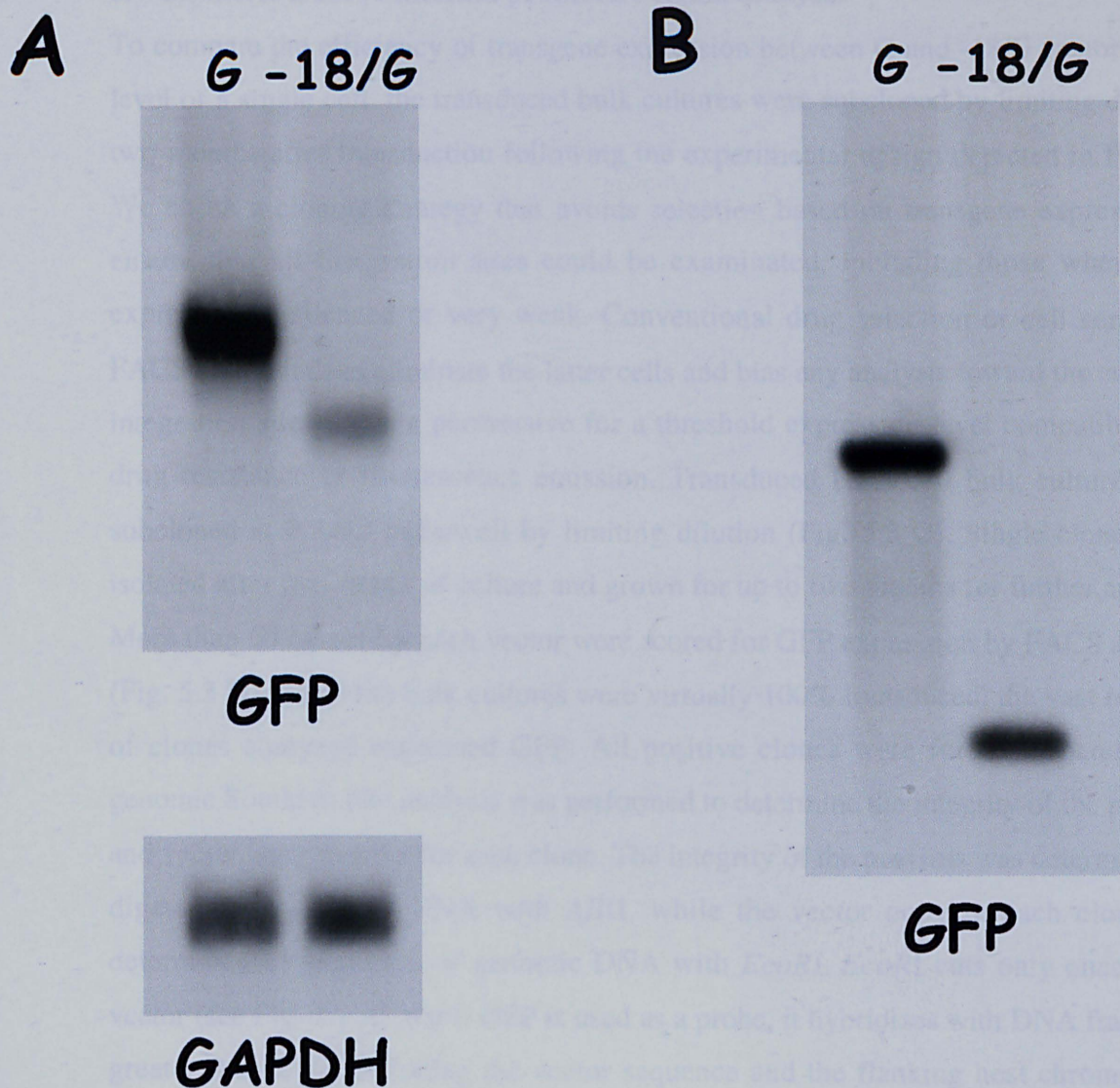


Figure 5.2. (A) Northern blot analysis of poly-A⁺ RNA extracted from HEL cells transduced with the G or the -18/G LV vectors and hybridized with GFP (upper panel) and GAPDH (lower panel) probes. (B) Southern blot analysis of genomic DNA extracted from HEL cell lines transduced with the G or the -18/G vector, digested with A₁/II and hybridized with a GFP probe.

5.4 Chimeric LTR vs internal promoter: clonal analysis

To compare the efficiency of transgene expression between G and -18/G vectors at the level of a single cell, the transduced bulk cultures were subcloned by limiting dilution, two months after transduction following the experimental design depicted in Fig. 5.3. We chose a cloning strategy that avoids selection based on transgene expression to ensure that all integration sites could be examined, including those where viral expression is silenced or very weak. Conventional drug selection or cell sorting by FACS would indeed eliminate the latter cells and bias any analysis toward the subset of integration sites that are permissive for a threshold expression level compatible with drug resistance or fluorescence emission. Transduced HEL cell bulk cultures were subcloned at 0.2-0.3 cells/well by limiting dilution (Fig. 5.3 C). Single clones were isolated after two weeks of culture and grown for up to two months for further analysis. More than 50 clones for each vector were scored for GFP expression by FACS analysis (Fig. 5.3 D). Since the bulk cultures were virtually 100% transduced, the vast majority of clones analyzed expressed GFP. All positive clones were further expanded and genomic Southern blot analysis was performed to determine the integrity of the provirus and vector copy number for each clone. The integrity of the provirus was determined by digestion of genomic DNA with *Afl*II, while the vector copy in each clone was determined by digestion of genomic DNA with *Eco*RI. *Eco*RI cuts only once in the vector (see Fig. 5.1 A) and if GFP is used as a probe, it hybridizes with DNA fragments greater than 2 kb, including the vector sequence and the flanking host chromosomal DNA (end-fragment analysis). In both groups of clones, end-fragment analysis with *Eco*RI showed no evidence of common or preferential sites of proviral integration (Fig. 5.3 E). In all clones tested, the proviral integrations ranged between 2 and 9 for the -18/G vector-derived clones, with a mean of 5.6, and from 4 to 17 for the G vector-derived clones, with a mean of 10.4. Since all selected clones of both groups carried more than one copy of integrated vector per cell, the single copy level of GFP expression was obtained by dividing all the integrations of each group by the number of clones analysed. The results showed a 2.6-fold increase in GFP expression level per integration in the clones derived from the G vector with respect to those derived from the -18/G vector (average of MFI/average of integration: 98.2 vs 37.9, respectively). A higher efficiency of GFP expression in G vector-derived clones was confirmed when we compared single clones for each group with the same, or a similar, number of integrations per cell (Fig. 5.4 B). GFP expression levels were consistently higher in

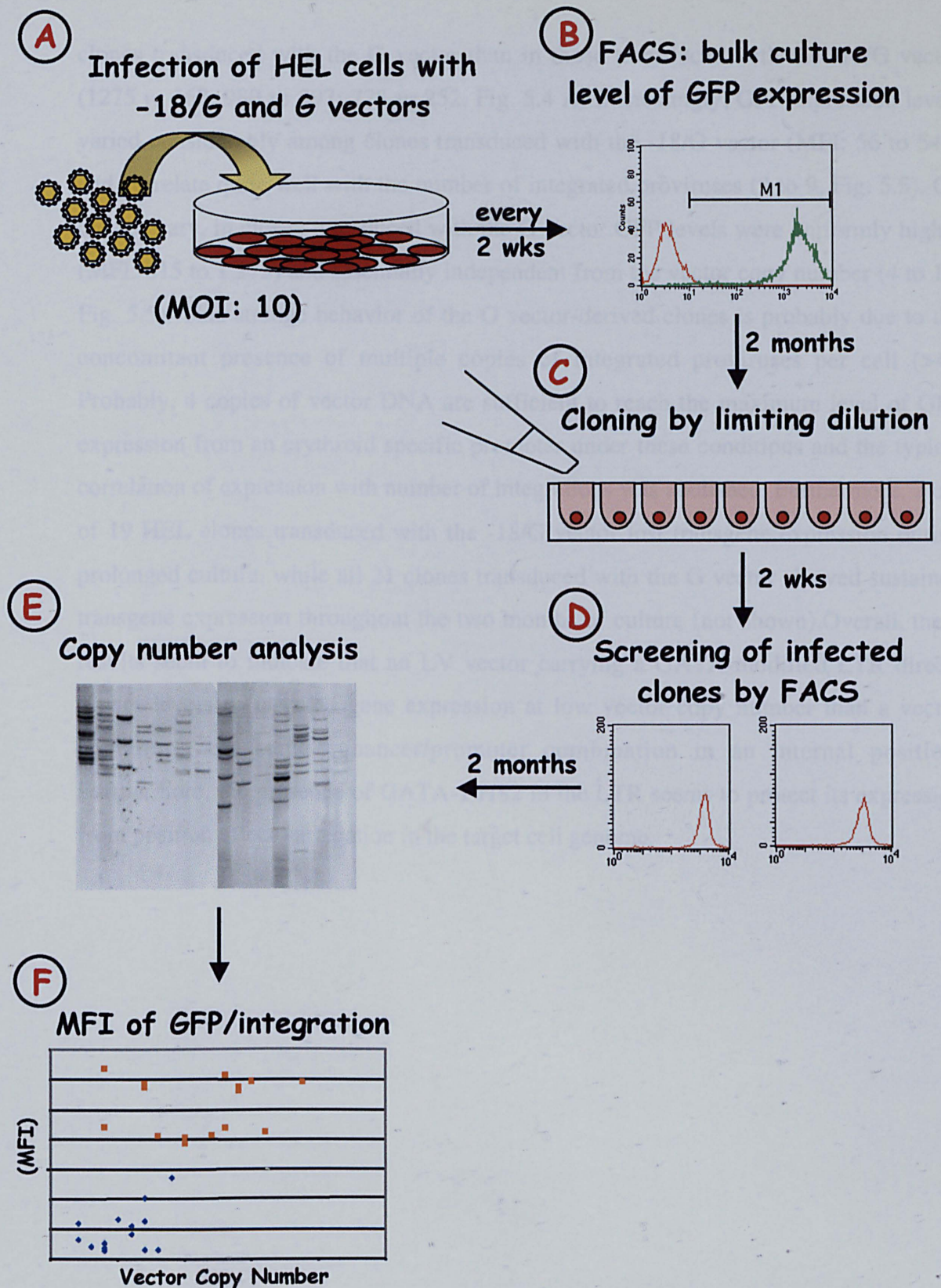


Figure 5.3. Experimental design. A large panel of HEL cell clones bearing -18/G and G vectors were generated and analyzed. HEL cells were infected with -18/G and G recombinant virions (A) and analyzed by FACS every week for two months (B). HEL cells were then subcloned (C) and scored for GFP expression by FACS (D). Vector copy number was determined by Southern blot analysis (E). Mean fluorescence intensity (MFI) of positive clones was determined by FACS analysis (F).

clones transduced with the G vector than in those transduced with the -18/G vector (1275 vs 168, 980 vs 397; 778 vs 252, Fig. 5.4 A). Interestingly, GFP expression levels varied considerably among clones transduced with the -18/G vector (MFI: 56 to 547) and correlate quite well with the number of integrated proviruses (2 to 9, Fig. 5.5). On the contrary, in clones transduced with the G vector GFP levels were uniformly higher (MFI: 815 to 1,275) and essentially independent from the vector copy number (4 to 17, Fig. 5.5). This strange behavior of the G vector-derived clones is probably due to the concomitant presence of multiple copies of integrated proviruses per cell (>4). Probably, 4 copies of vector DNA are sufficient to reach the maximum level of GFP expression from an erythroid specific promoter under these conditions and the typical correlation of expression with number of integrations was abolished. Furthermore, 2 out of 19 HEL clones transduced with the -18/G vector lost transgene expression during prolonged culture, while all 21 clones transduced with the G vector showed sustained transgene expression throughout the two months of culture (not shown). Overall, these results seem to indicate that an LV vector carrying a GATA-modified LTR directs higher levels of targeted gene expression at low vector copy number than a vector containing the same enhancer/promoter combination in an internal position. Furthermore, the presence of GATA-1 HS2 in the LTR seems to protect its expression from position effect variegation in the target cell genome.

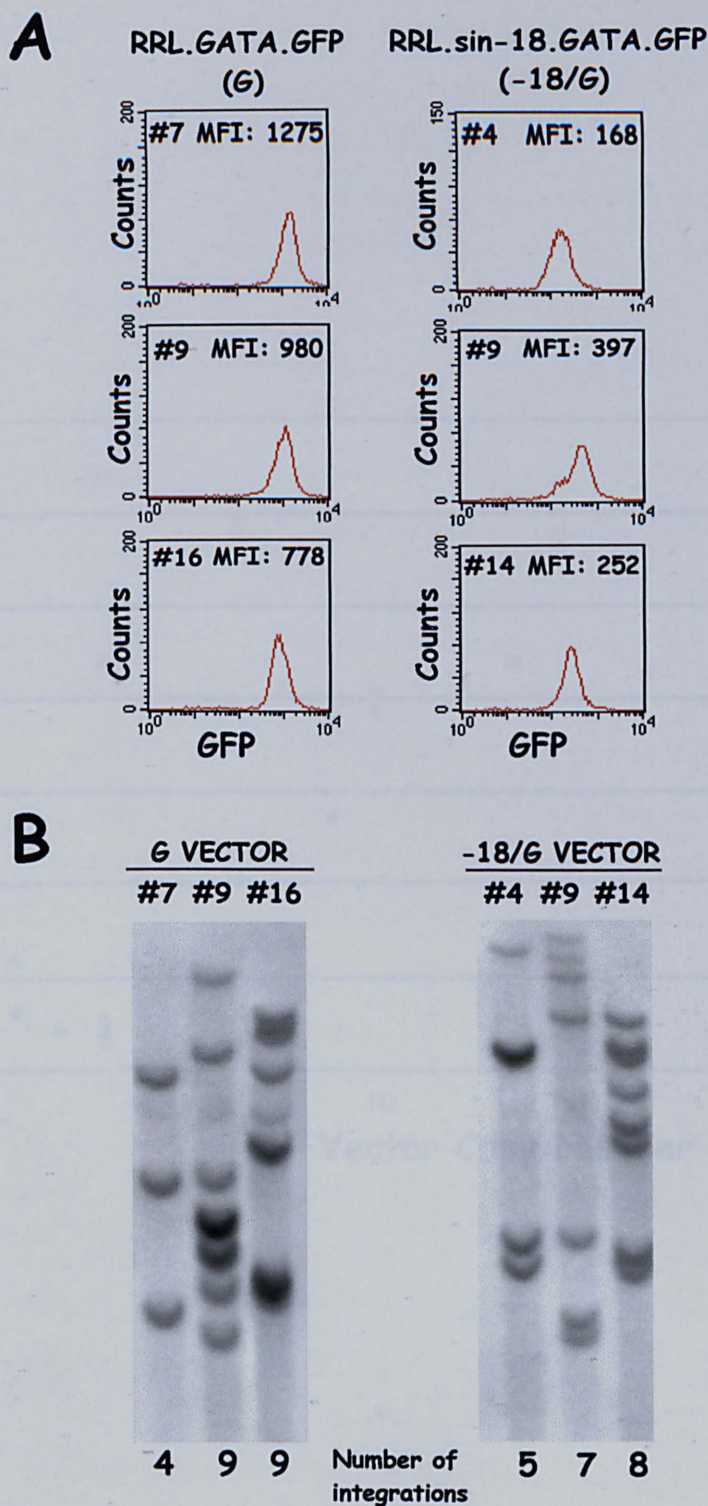


Figure 5.4. (A) Single-channel flow cytometry of GFP expression in HEL cell clones transduced with the G (left panel) or the -18/G (right panel) vectors and analyzed 2 months after transduction. Distribution of transduced cells are indicated by red peaks. Mean fluorescence intensity (MFI) of GFP expression is in arbitrary units. (B) Southern blot analysis of genomic DNA extracted from HEL clones transduced with the G or the -18/G vector, digested with *EcoRI*, and hybridized to a GFP probe. The number of proviral integrations is reported for each clone.

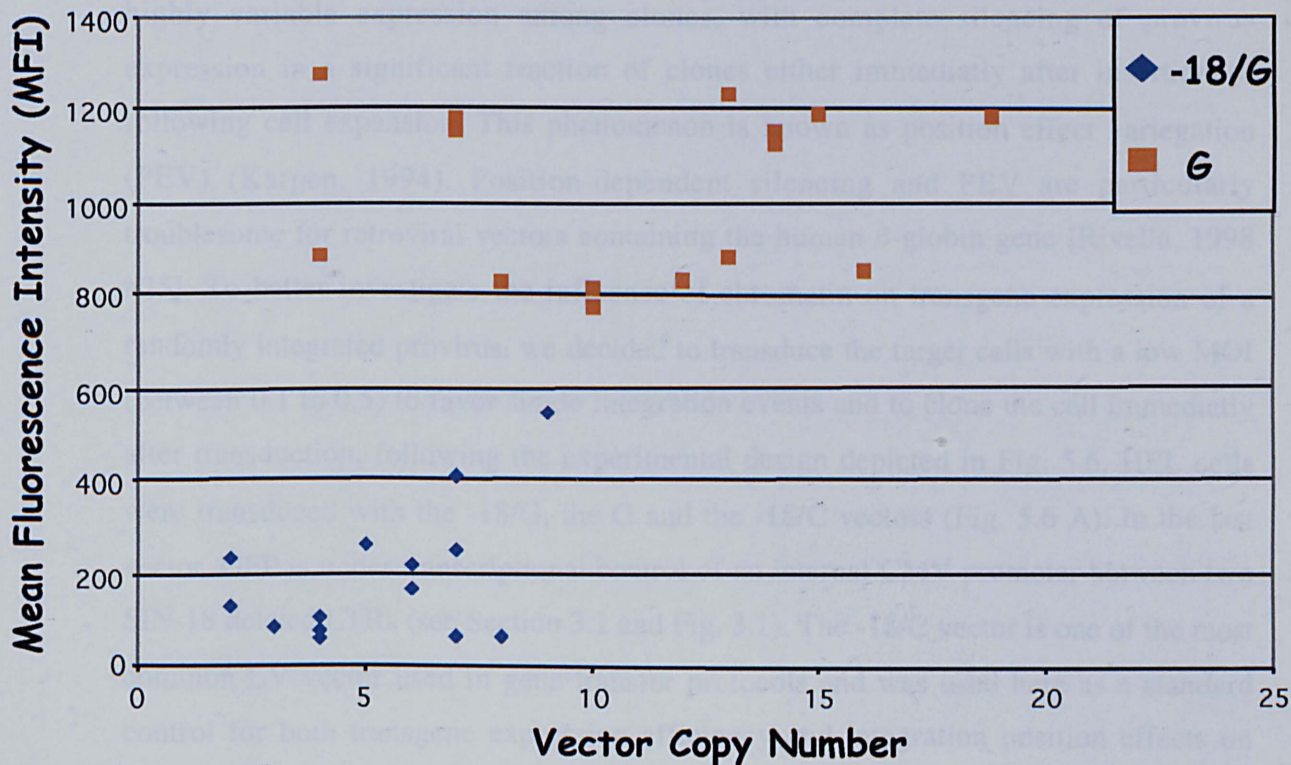


Figure 5.5. Mean fluorescence intensity (MFI) of -18/C (blue dots) and G (orange squares) vector-derived clones was determined by FACS analysis. MFI values (Y axis), expressed as arbitrary units, were plotted versus vector copy number determined by Southern blot analysis (X axis).

Transduction of HEL cells with a relatively high MOI allowed us to compare transgene expression efficiency of -18/G and G vectors starting from fully transduced, highly expressing bulk cultures. But, with this experimental design, due to the concomitant presence of multiple copies of the vector in all clones analyzed, we lost all information regarding chromatin position effects on transgene expression of a single vector clone. Position effects reflect the long-range influence of chromatin structure and may silence transcription or alter its efficiency. The result of this chromatin influence can lead to highly variable expression among clones, with complete silencing of provirus expression in a significant fraction of clones either immediately after insertion or following cell expansion. This phenomenon is known as position effect variegation (PEV) (Karpen, 1994). Position-dependent silencing and PEV are particularly troublesome for retroviral vectors containing the human β -globin gene [Rivella, 1998 #25]. To better investigate the influence of chromatin on transgene expression of a randomly integrated provirus, we decided to transduce the target cells with a low MOI (between 0.1 to 0.5) to favor single integration events and to clone the cell immediately after transduction, following the experimental design depicted in Fig. 5.6. HEL cells were transduced with the -18/G, the G and the -18/C vectors (Fig. 5.6 A). In the last vector, GFP is under transcriptional control of an internal CMV promoter between two SIN-18 deleted LTRs (see Section 3.1 and Fig. 3.1). The -18/C vector is one of the most common LV vector used in gene transfer protocols and was used here as a standard control for both transgene expression efficiency and integration position effects on transgene expression. Transduced HEL cells were maintained as bulk cultures and transduction efficiency was determined by FACS analysis of GFP expression four days after transduction (Fig. 5.7). Transduction efficiency was very similar between the three different bulk populations: 48%, 53% and 44% for G, -18/G and -18/C vectors, respectively (Fig. 5.7). HEL cells were therefore subcloned at 0.3 cell/well by limiting dilution a few days after transduction (Fig. 5.6 C). Single clones were isolated after two weeks of culture and grown for up to two weeks for further analysis. For each vector more than 50 clones were scored by FACS analysis for GFP expression (Fig. 5.6 D). Among them, all those clones that were negative for GFP expression, determined by FACS analysis, were further expanded and scored for vector integration by PCR analysis (Fig. 5.6 E). Vector DNA positive clones were further analyzed by Southern blotting to determine the integrity of the provirus and vector copy number, as previously described (Fig. 5.6 F). Results obtained by PCR and confirmed by Southern blot

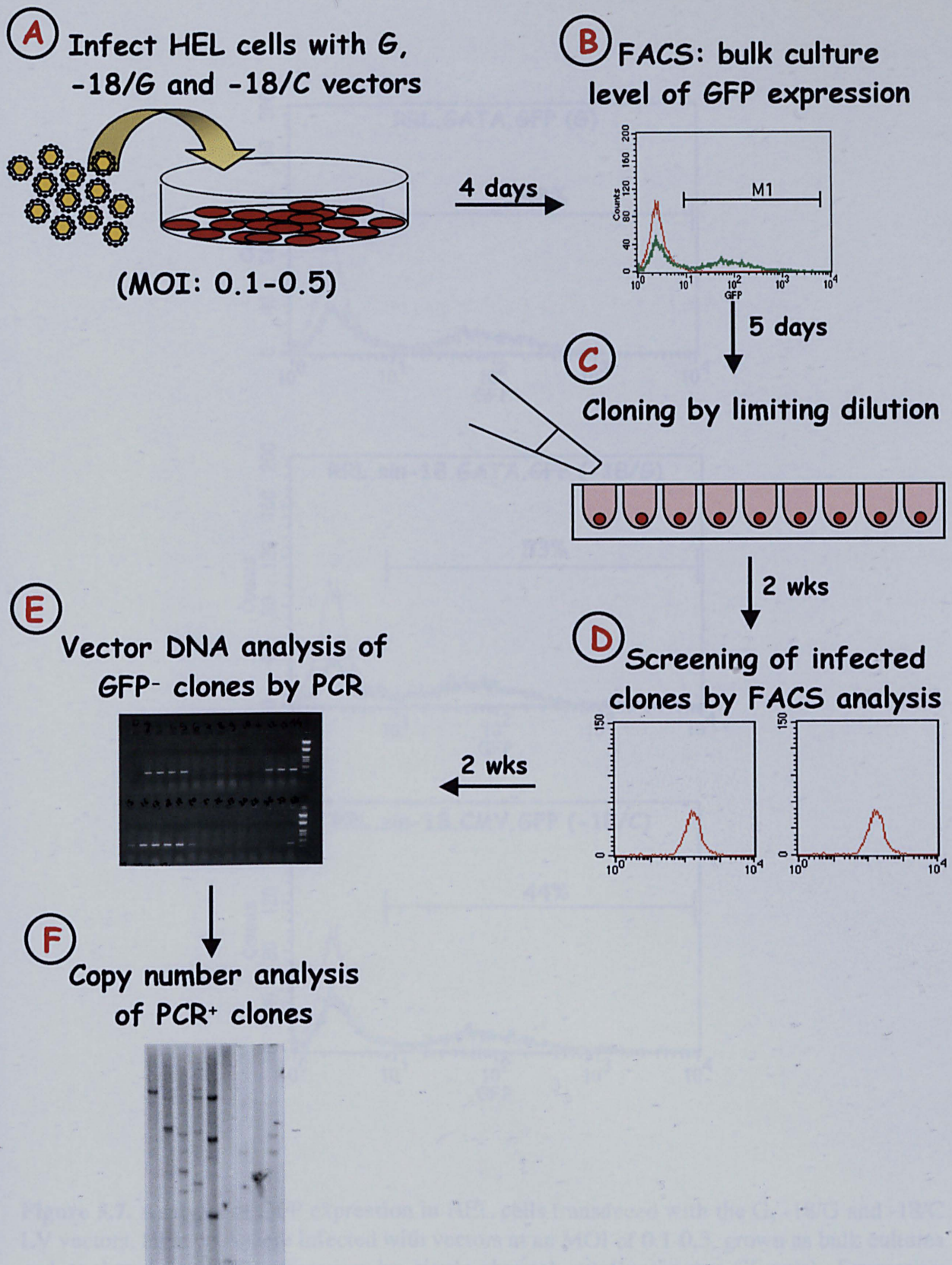


Figure 5.6. Experimental design. A large panel of HEL cell clones bearing -18/G, G and -18/C vectors were generated and analyzed. HEL cells were infected at low MOI (0.1-0.5) with -18/G, G and -18/C recombinant virions (A) and analyzed by FACS (B). HEL cells were then subcloned 5 days after transduction (C) and scored for GFP expression by FACS (D). GFP negative clones were analyzed by PCR (E) and copy number in PCR positive clones was determined by Southern blot analysis (F).

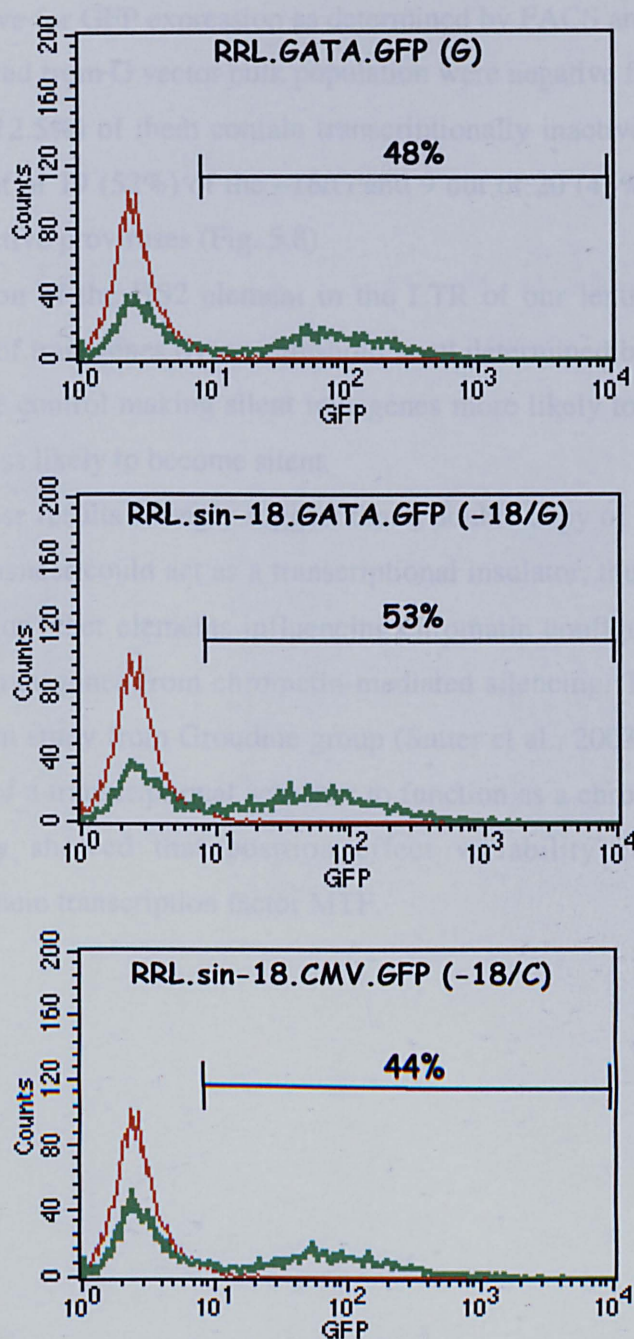


Figure 5.7. Analysis of GFP expression in HEL cells transduced with the G, -18/G and -18/C LV vectors. HEL cells were infected with vectors at an MOI of 0.1-0.5, grown as bulk cultures and analyzed for GFP expression by single-channel cytofluorimetry (X axis). Expression profiles are indicated by a red (mock infected cells) or a green (infected cells) line respectively. Percentages of GFP expressing cells are indicated within each panel.

analysis are shown in Fig. 5.8. Analysis of those G, -18/G and -18/C cell clones that were negative for GFP expression as determined by FACS analysis are illustrated. Most clones derived from G vector bulk population were negative for vector DNA, and only 3 out of 24 (12.5%) of them contain transcriptionally inactive proviruses. On the other hand, 10 out of 19 (52%) of the -18/G and 9 out of 20 (45%) of the -18/C cell clones contain inactive proviruses (Fig. 5.8).

The insertion of the HS2 element in the LTR of our lentiviral vector enhances the expression of transgenes over a threshold level determined by the expression profile of the negative control making silent transgenes more likely to become active and active transgenes less likely to become silent.

Overall, these results strongly suggest that a double copy of the GATA-HS2 erythroid-specific enhancer could act as a transcriptional insulator, thus replacing LCRs, matrix-attachment or other elements influencing chromatin configuration to shield randomly integrated transgenes from chromatin-mediated silencing. These results are in partial with a recent study from Groudine group (Sutter et al., 2003). In this paper they tested the ability of a transcriptional activator to function as a chromatin insulator in cultured cells. They showed that position effect variability can be suppressed by a metallothionein transcription factor MTF.

5.1 Expression of two independently regulated transcriptional units

One of the possible advantages of our breeding strategy is that the transient transduction

GFP⁻ clones (FACS analysis)

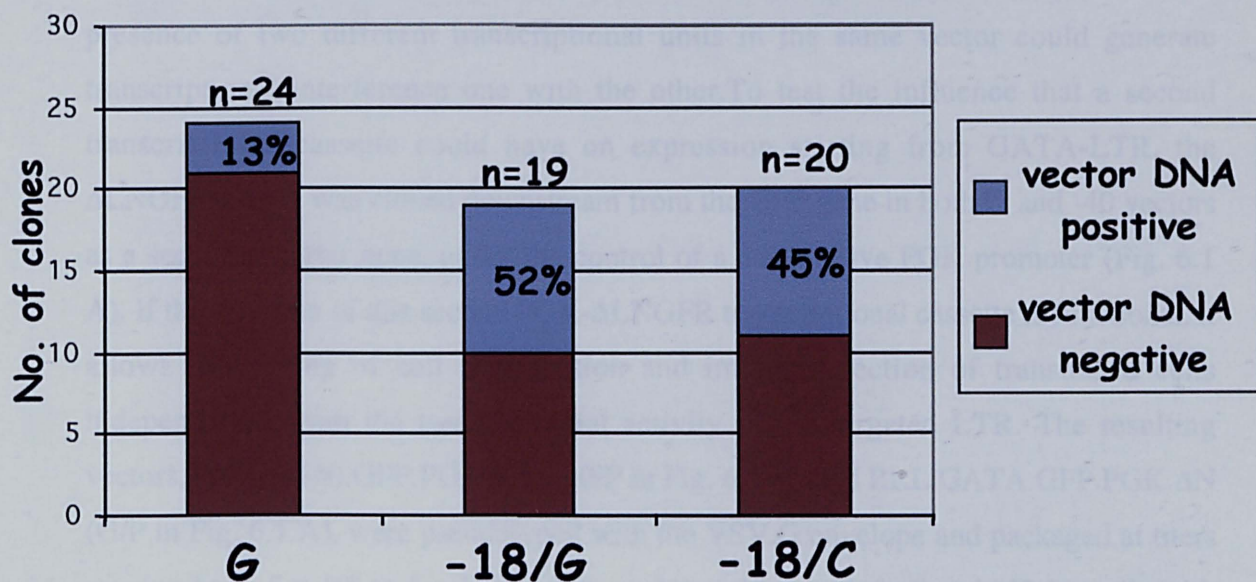


Figure 5.8. G, -18/G and -18/C cell clones negative for GFP expression by FACS analysis were scored for vector DNA integration by PCR and Southern blot analysis 5 weeks after transduction. 3 out of 24 (13%) of G, 10 out of 19 (52%) of -18/G and 9 out of 20 (45%) -18/C GFP⁻ clones were positive for vector DNA.

Chapter 6

Optimising transfer vector design.

6.1 Expression of two independently regulated transcriptional units.

One of the possible advantages of our targeting strategy is that the internal transcription unit can be used to express a second gene providing an additional function, for example an *in vivo* selectable marker, generating a bi-functional LV vector. However, the presence of two different transcriptional units in the same vector could generate transcriptional interference one with the other. To test the influence that a second transcriptional cassette could have on expression starting from GATA-LTR, the Δ LNGFR cDNA was cloned downstream from the GFP gene in both G and -40 vectors as a second reporter gene, under the control of a constitutive PGK promoter (Fig. 6.1 A). If the insertion of this second PGK- Δ LNGFR transcriptional cassette is functional, it allows monitoring of cell transduction and immunoselection of transduced cells independently from the transcriptional activity of the targeted LTR. The resulting vectors, RRL.sin-40.GFP.PGK. Δ N (-40/P in Fig. 6.1 A) and RRL.GATA.GFP.PGK. Δ N (G/P in Fig. 6.1 A), were pseudotyped with the VSV.G envelope and packaged at titers ranging from 5×10^6 to 5×10^7 TU/ml, as determined by infection of HeLa cells with serial dilutions of viral supernatants and FACS analysis of Δ LNGFR expression (Fig. 6.1 B). Dilutions of G vector viral supernatant started from 10^{-2} up to 10^{-3} . Transduction efficiency was determined by FACS analysis of GFP expression (Fig. 6.1 B). Viral titer was calculated at a dilution of 10^{-3} and yielded a titer of 2×10^7 TU/ml. To study the expression characteristics of the two transcriptional units, human myeloblastic (Kasumi-1), myelomonocytic (U937), erythroblastic (K562 and HEL) and non-hematopoietic (HeLa) cell lines as well as murine erythroblastic (MEL) and fibroblastic (NIH-3T3) cell lines were infected with -40/P and G/P vectors (MOI: 5-10) and grown as bulk cultures. Transduction efficiency ranged between 67 and 96%, depending on the cell line, as determined by FACS analysis of LNGFR expression.

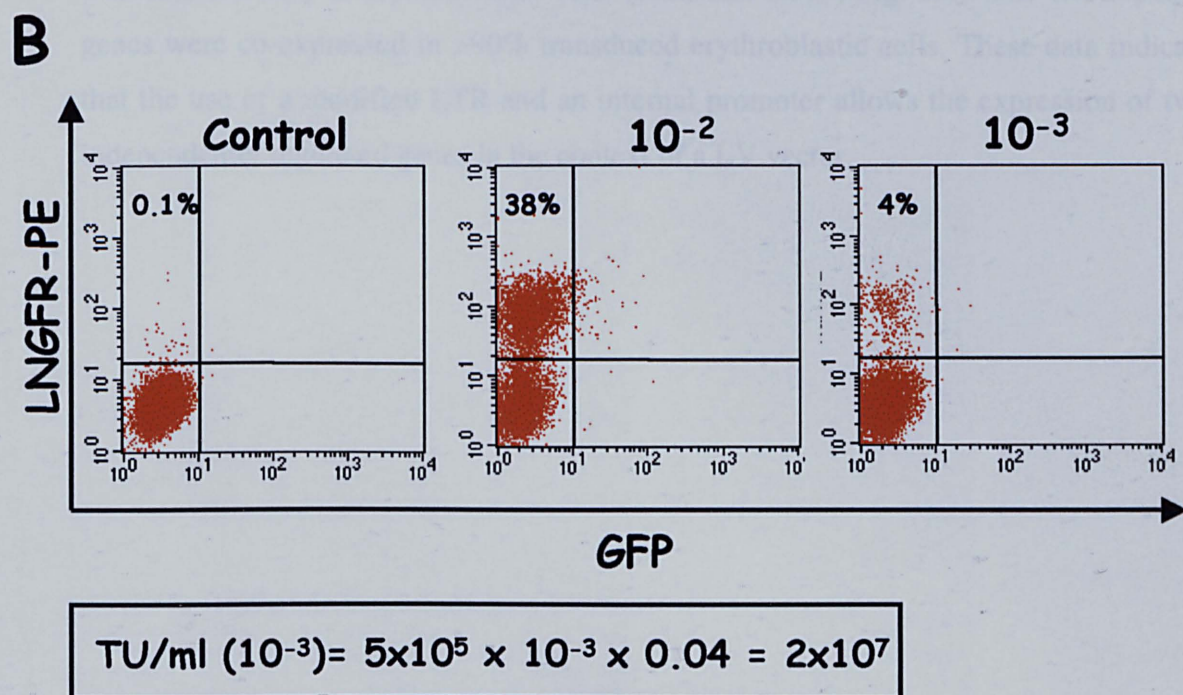
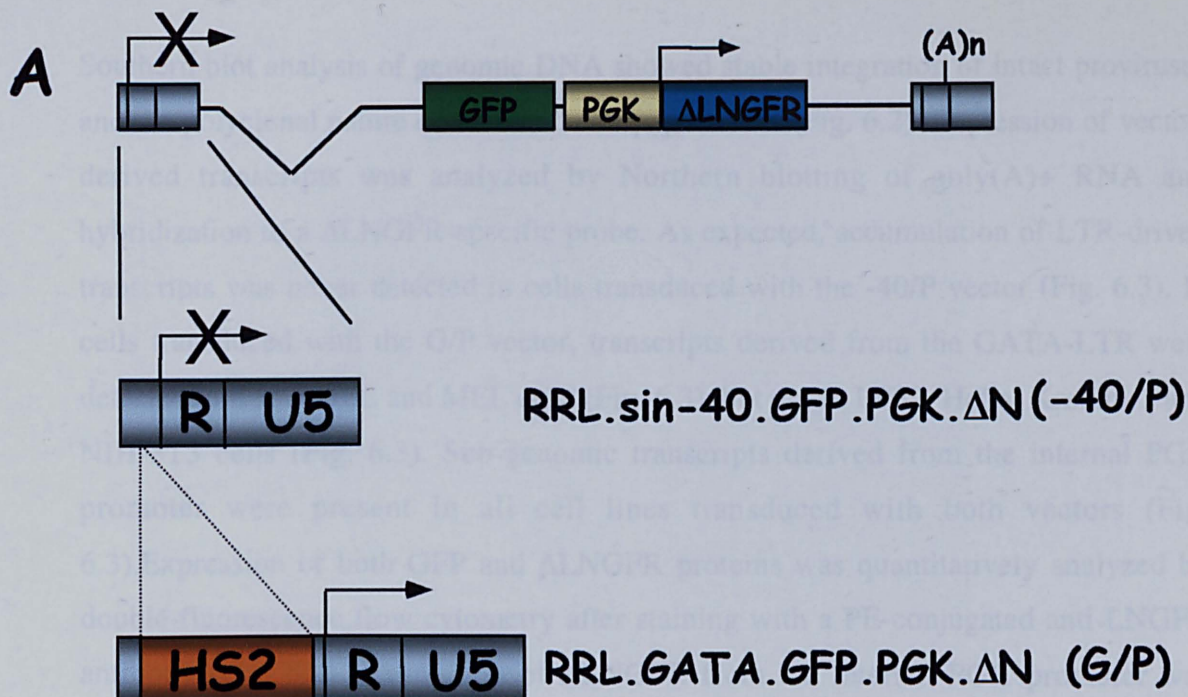


Figure 6.1. (A) Schematic map of the RRL.sin-40.GFP.PGK.ΔN (-40/P) and the RRL.GATA.GFP.PGK.ΔN (G/P) LV vectors. In the -40/P vector, the LTR carries a -418 to -40 deletion removing the U3 enhancer, while in the G/P vector the same region is replaced by the GATA-1 HS2 auto regulatory enhancer. The arrows indicate the transcription start sites of the LTR or the internal phosphoglycerokinase (PGK) promoter. (B) End-point titration of the G/P vector viral supernatant in HeLa cells. HeLa cells transduced with serial dilutions of the viral supernatant (10^{-2} - 10^{-3}) were grown as bulk cultures and analyzed for GFP expression (X axis) and LNGFR expression (Y-axis) by FACS. Percentages of LNGFR expressing cells are indicated within each quadrant. Viral titer was calculated at a dilution of 10^{-3} as indicated.

Southern blot analysis of genomic DNA showed stable integration of intact proviruses and the polyclonal nature of all tested cell populations (Fig. 6.2). Expression of vector-derived transcripts was analyzed by Northern blotting of poly(A)⁺ RNA and hybridization to a Δ LNGFR-specific probe. As expected, accumulation of LTR-driven transcripts was never detected in cells transduced with the -40/P vector (Fig. 6.3). In cells transduced with the G/P vector, transcripts derived from the GATA-LTR were detected in K562, HEL and MEL cells (Fig. 6.3), but not in U937, HeLa, Kasumi-1 and NIH-3T3 cells (Fig. 6.3). Sub-genomic transcripts derived from the internal PGK promoter were present in all cell lines transduced with both vectors (Fig. 6.3). Expression of both GFP and Δ LNGFR proteins was quantitatively analyzed by double-fluorescence flow cytometry after staining with a PE-conjugated anti-LNGFR antibody (Fig. 6.4). Expression of Δ LNGFR from the internal PGK promoter was observed in all transduced cell lines, while expression of GFP from the GATA-LTR was detected only in erythroblastic cells (HEL and K562, Fig. 6.4). The two reporter genes were co-expressed in >90% transduced erythroblastic cells. These data indicate that the use of a modified LTR and an internal promoter allows the expression of two independently regulated genes in the context of a LV vector.

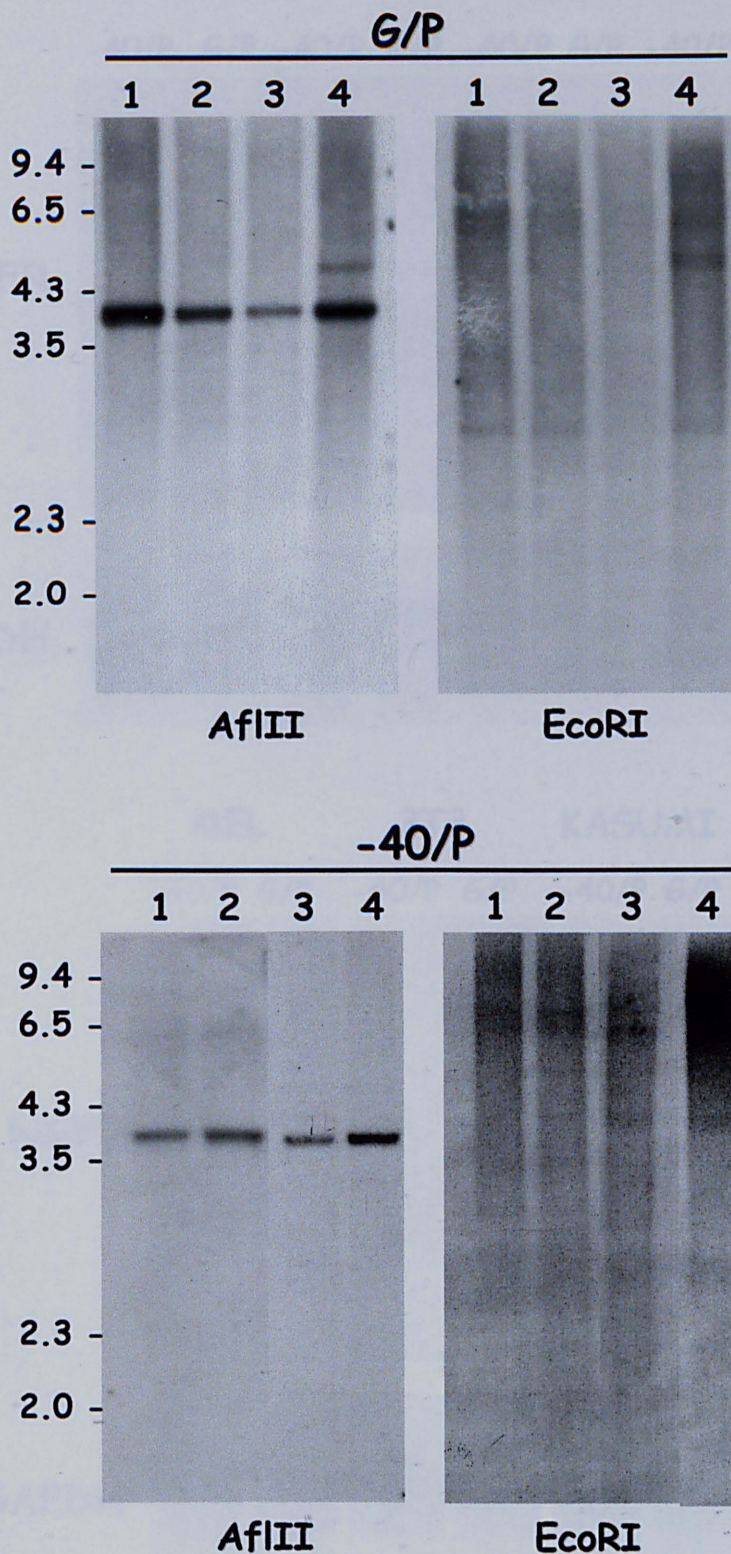


Figure 6.2. Southern blot analysis of genomic DNA extracted from K562 (1), HEL (2), HeLa (3) and U937 (4) cell lines transduced with the G/P (upper panels) or the -40/P (lower panels) vectors, digested with *AflIII* or *EcoRI* and hybridised with a GFP probe. Molecular size markers are indicated in kilobases on the left.

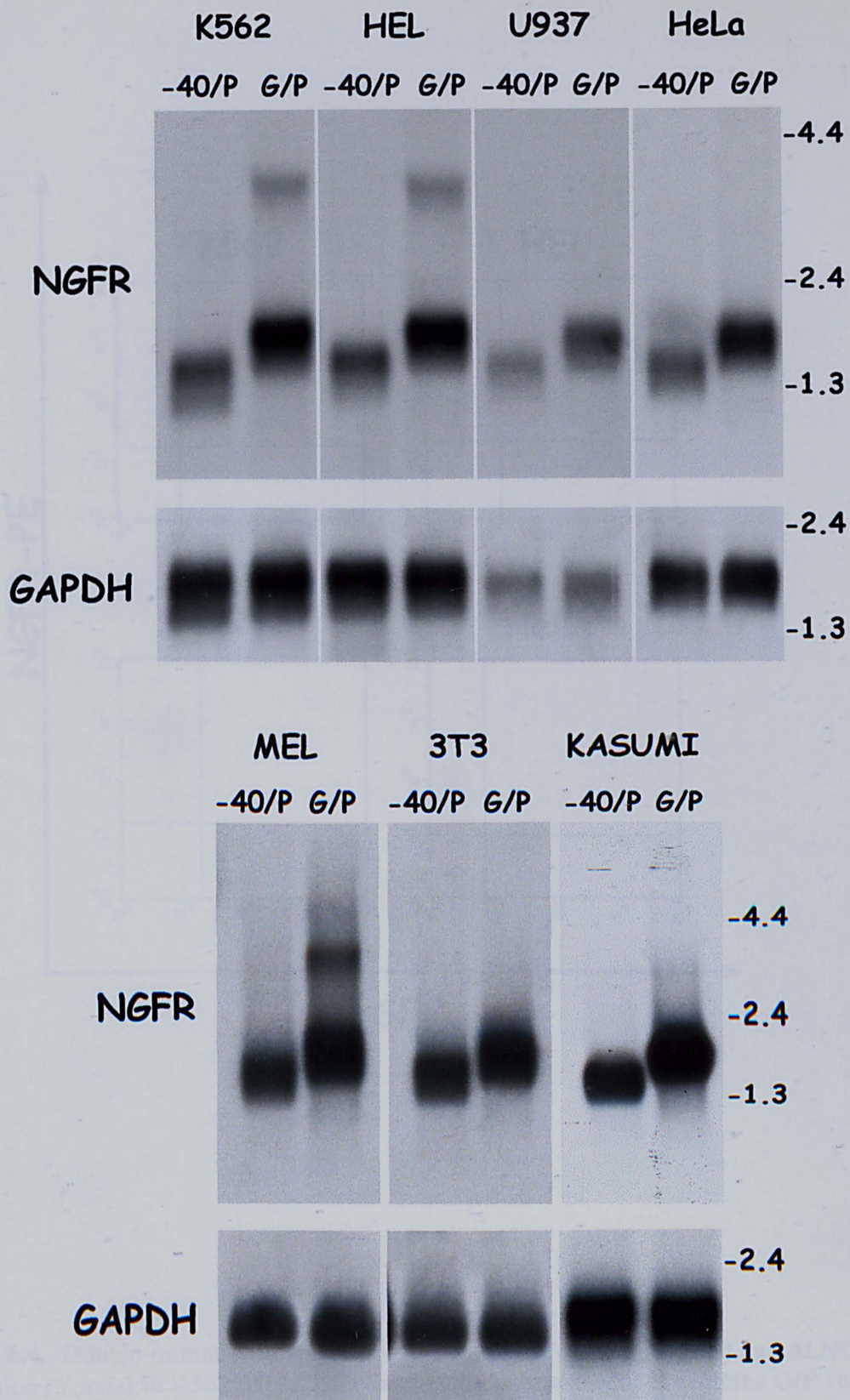


Fig 6.3. Northern blot analysis of poly(A)⁺ RNA extracted from K562, HEL, MEL (erythroblastic), U937 and Kasumi-1 (myeloblastic), HeLa and NIH-3T3 (non-hematopoietic) cell lines transduced with the -40/P or the G/P vector and hybridized with NGFR (upper panels) and GAPDH (lower panels) probes. Molecular weight markers (in kilobases) are indicated on the left.

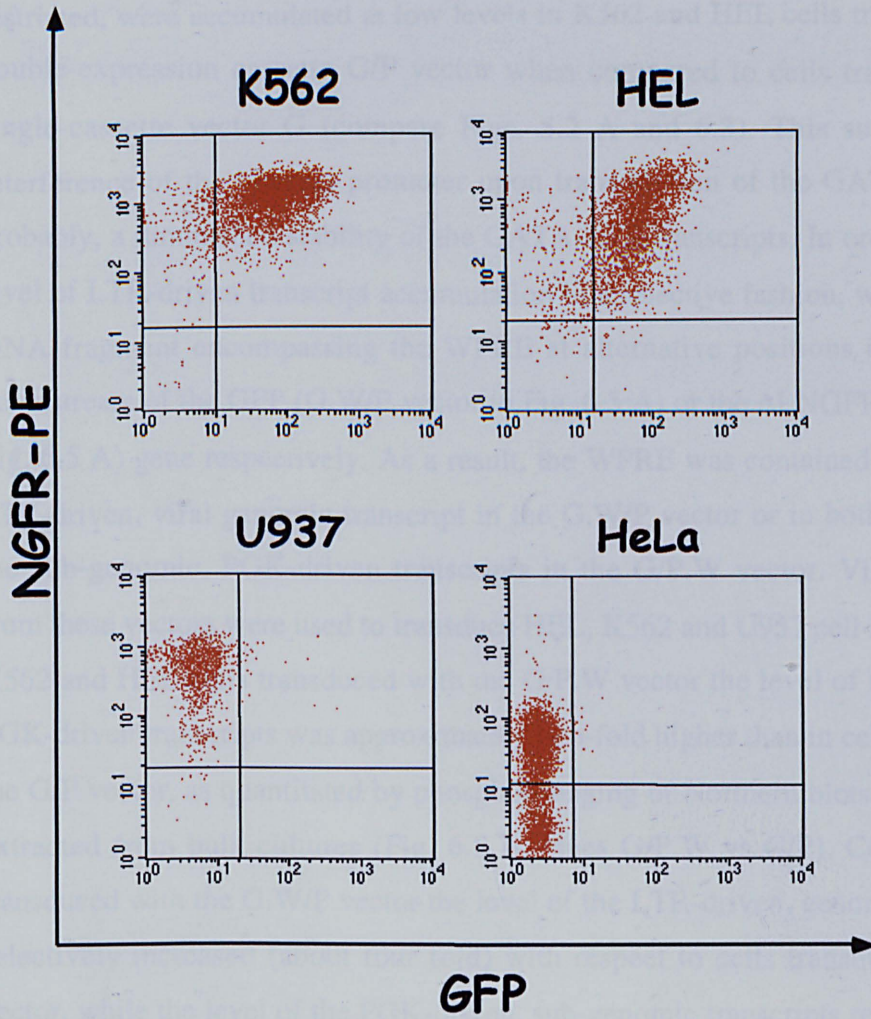


Figure 6.4. Double-immunofluorescence FACS analysis of GFP (X axis) and Δ LNGFR expression (Y axis) in K562, HEL, U937 and HeLa cells transduced with the G/P vector (see Fig. 3.11) at a MOI of 10. Cells were grown as bulk culture and analyzed 3 days after infection after staining with a PE-conjugated anti-LNGFR antibody.

6.2 Selective enhancement of transgene expression by specific positioning of WPRE.

Northern blot analysis showed that transcripts from the modified LTR, although restricted, were accumulated at low levels in K562 and HEL cells transduced with the double-expression cassette G/P vector when compared to cells transduced with the single-cassette vector G (compare Figs. 5.2 A and 6.3). This suggests a negative interference of the internal promoter upon transcription of the GATA-LTR, or more probably, a diminished stability of the GATA-LTR transcripts. In order to improve the level of LTR-driven transcript accumulation in a selective fashion, we cloned a 500-bp DNA fragment encompassing the WPRE at alternative positions in the G/P vector, downstream of the GFP (G.W/P vector in Fig. 6.5 A) or the Δ LNGFR (G/P.W vector in Fig. 6.5 A) gene respectively. As a result, the WPRE was contained exclusively in the LTR-driven, viral genomic transcript in the G.W/P vector or in both the genomic and the sub-genomic, PGK-driven transcripts in the G/P.W vector. Viral stocks derived from these vectors were used to transduce HEL, K562 and U937 cell lines (MOI: 10). In K562 and HEL cells transduced with the G/P.W vector the level of both the LTR- and PGK-driven transcripts was approximately two-fold higher than in cells transduced with the G/P vector, as quantitated by phosphorimaging of Northern blots of poly(A)+ RNA extracted from bulk cultures (Fig. 6.5 B, lanes G/P.W vs G/P). Conversely, in cells transduced with the G.W/P vector the level of the LTR-driven, genomic transcripts was selectively increased (about four fold) with respect to cells transduced with the G/P vector, while the level of the PGK-driven, sub-genomic transcripts remained essentially unchanged (Fig. 6.5 B, lanes G.W/P vs G/P). Interestingly, addition of the WPRE in either configuration did not rescue accumulation of genomic transcripts in non-erythroid cells (U937 in Fig 6.5 B) and had therefore no effect on the transcriptional targeting of the GATA-LTR. Protein expression data paralleled the results obtained at the level of RNA accumulation (Fig. 6.6). PGK-driven, Δ LNGFR expression increased slightly in both HEL and K562 cells transduced with the G/P.W vector compared to cells transduced with the G/P and the G.W/P vectors (MFI: 1,336 vs 759 and 1,051 respectively in HEL cells, and 1345 vs 1,010 and 1,116 respectively in K562 cells, in a representative experiment, see Fig. 6.6). Conversely, GFP expression increased three- to five-fold in HEL and K562 cells transduced with the G/P.W vector compared to cells transduced with G/P and the G.W/P (MFI: 345 vs 78 and 72 respectively in HEL cells, and 260 vs 68 and 107 respectively in K562 cells, see Fig. 6.6). Transduced U937 cells

A

RRL.sin-40.GFP.Wpre.PGK. Δ N (G.W/P)



RRL.sin-40.GFP.PGK. Δ N.Wpre (G/P.W)

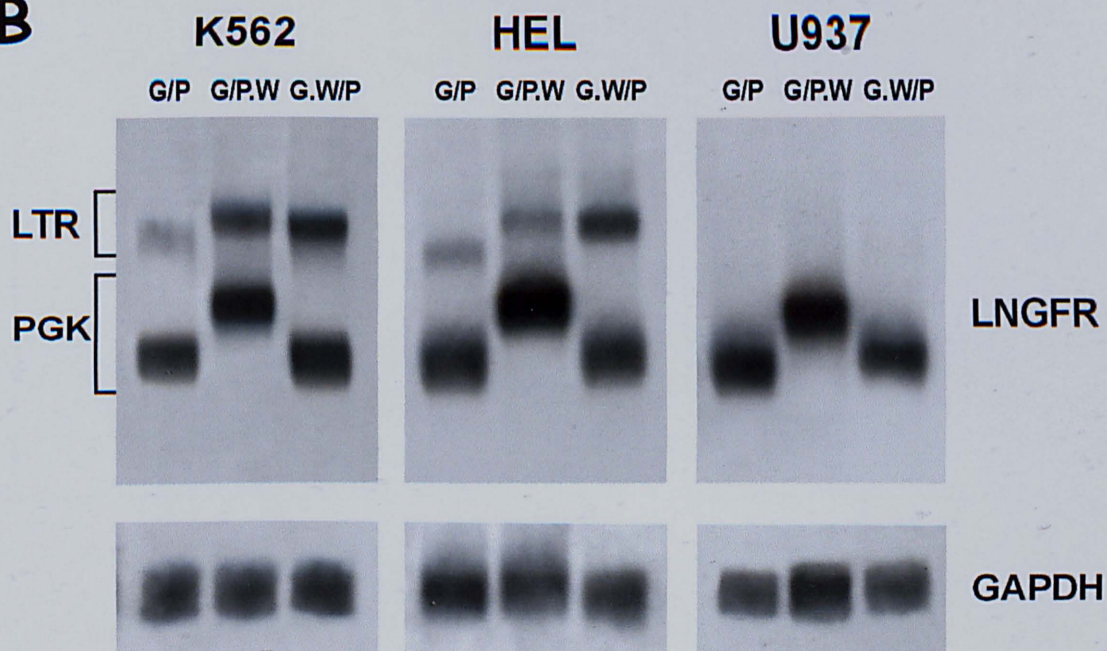
**B**

Figure 6.5. (A) Schematic map of the RRL.GATA.GFP.PGK. Δ N.W (G/P.W) and RRL.GATA.GFP.W.PGK. Δ N (G.W/P) vectors in their proviral forms. The woodchuck hepatitis virus post-transcriptional regulatory element (W) is inserted at alternative positions in the vectors, both derived from the G/P vector described in Fig 311A. (B) Northern blot analysis of poly(A)⁺ RNA extracted from K562, HEL, and U937 cells transduced with the G/P, G/P.W, or G.W/P vectors and hybridized with LNGFR (upper panel) or GAPDH (lower panel) probes. Transcripts originating from the viral LTR or the internal PGK promoter are indicated on the left.

never showed expression of GFP, while Δ LNFR levels increased slightly in cells transduced with G/P.W (MFI: 422) with respect to cells transduced with G/P or G.W/P (MFI: 318 and 303 respectively, see Fig. 6.6). These results demonstrate that the presence of WPRE within a transcript selectively enhances its accumulation in transduced cells, thus allowing differential modulation of the expression levels of two different genes carried by the same lentiviral vector. The G.W/P vector was therefore chosen to evaluate transcriptional targeting of the GATA-LTR in human primary HSCs.

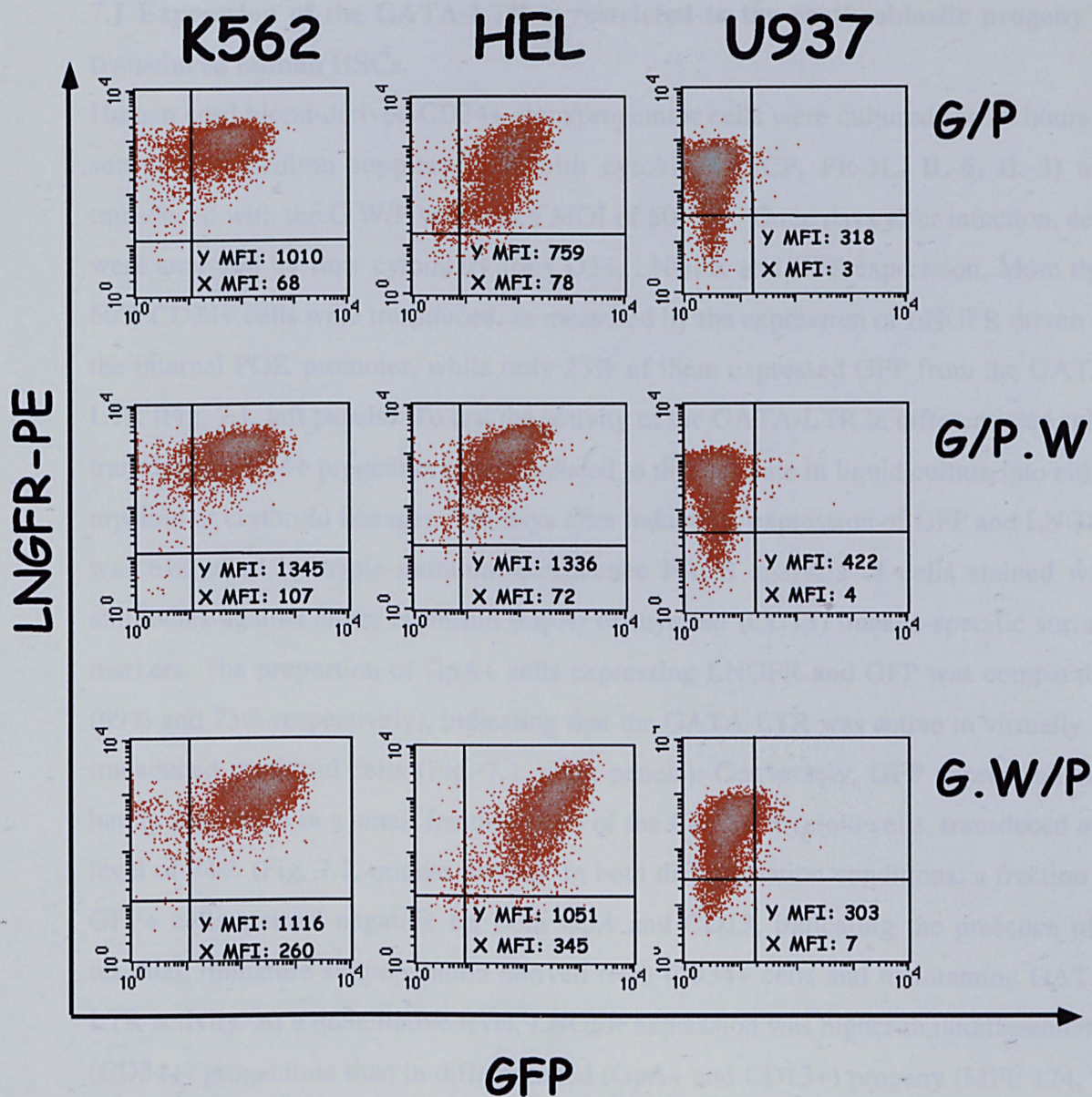


Figure 6.6. Double-immunofluorescence FACS analysis of GFP (X axis) and Δ LNGFR expression (Y axis) in K562, HEL, U937 cells transduced with the G/P, G/P.W and G.W/P vectors (see Fig. 6.1A and 6.3A). Cells were grown as bulk culture and analyzed 4 days after infection after staining with a PE-conjugated anti-LNGFR antibody. Mean fluorescence intensity of GFP (X MFI) and of NGFR expression (Y MFI) are in arbitrary units.

Chapter 7

Activity of GATA-LTR vector in HSCs.

7.1 Expression of the GATA-LTR is restricted to the erythroblastic progeny of transduced human HSCs.

Human cord blood-derived CD34⁺ stem/progenitor cells were cultured for 24 hours in serum-free medium supplemented with cytokines (SCF, Flt-3L, IL-6, IL-3) and transduced with the G.W/P vector at a MOI of 50-100. Three days after infection, cells were analyzed by flow cytometry for CD34, LNGFR and GFP expression. More than 60% CD34⁺ cells were transduced, as measured by the expression of LNGFR driven by the internal PGK promoter, while only 23% of them expressed GFP from the GATA-LTR (Fig. 7.1, left panels). To test the activity of the GATA-LTR in differentiated cells, transduced CD34⁺ progenitors were induced to differentiate in liquid culture into either myeloid or erythroid lineages. Ten days after induction, expression of GFP and LNGFR was evaluated by triple-immunofluorescence FACS analysis of cells stained with antibodies against either erythroid (GpA) or myeloid (CD13) lineage-specific surface markers. The proportion of GpA⁺ cells expressing LNGFR and GFP was comparable (69% and 73% respectively), indicating that the GATA-LTR was active in virtually all transduced erythroid cells (Fig. 7.1, right panels). Conversely, GFP expression was barely detectable in a small fraction (9%) of the CD13⁺ myeloid cells, transduced at a level of 74% (Fig. 7.1, middle panels). In both differentiation conditions, a fraction of GFP⁺ cells stained negative for both GpA and CD13, indicating the presence of a residual, immature subpopulation derived from CD34⁺ cells and maintaining GATA-LTR activity. At a quantitative level, LNGFR expression was higher in undifferentiated (CD34⁺) progenitors than in differentiated (GpA⁺ and CD13⁺) progeny (MFI: 174, 58 and 68 respectively), whereas GFP increased five-fold in the GpA⁺ progeny of CD34⁺ cells (MFI: from 52 to 255), showing that expression from the GATA-LTR increases with erythroid cell differentiation, and suggesting that in an appropriate cell context the activity of a targeted LTR is higher than that of a constitutive internal promoter.

To analyze transgene expression at the level of single colonies, CD34⁺ cells were transduced with G.W/P vector, immunoselected for Δ LNGFR expression by immunomagnetic cell sorting and directly scored for GFP expression by fluorescence microscopy in clonal methylcellulose cultures. The introduction of the immunoselection step allowed us to analyze GATA-LTR-driven gene expression only in cells

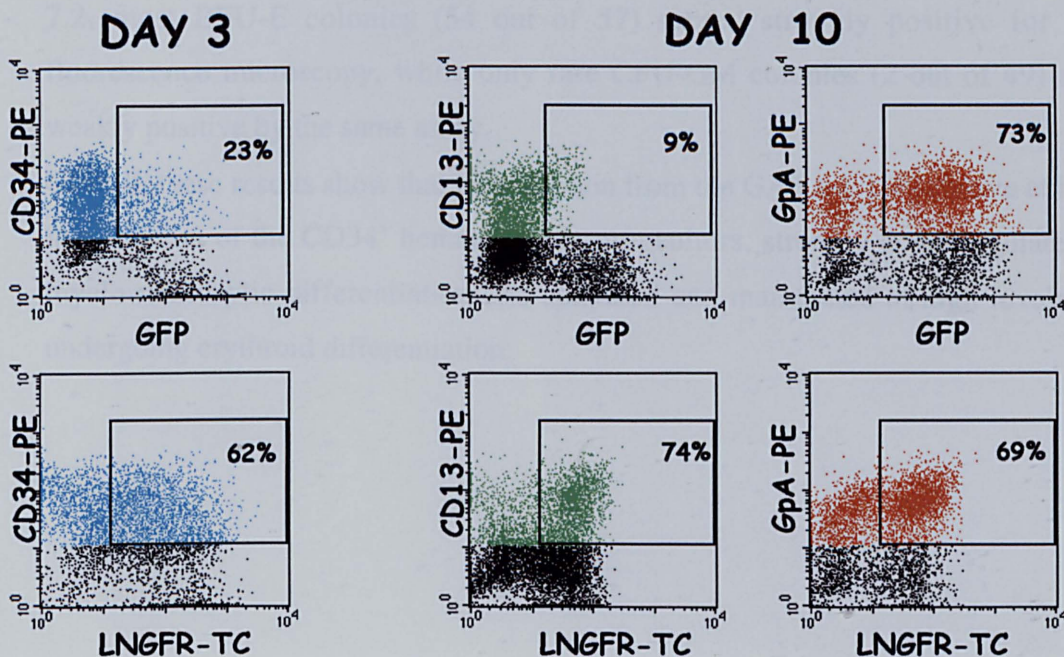
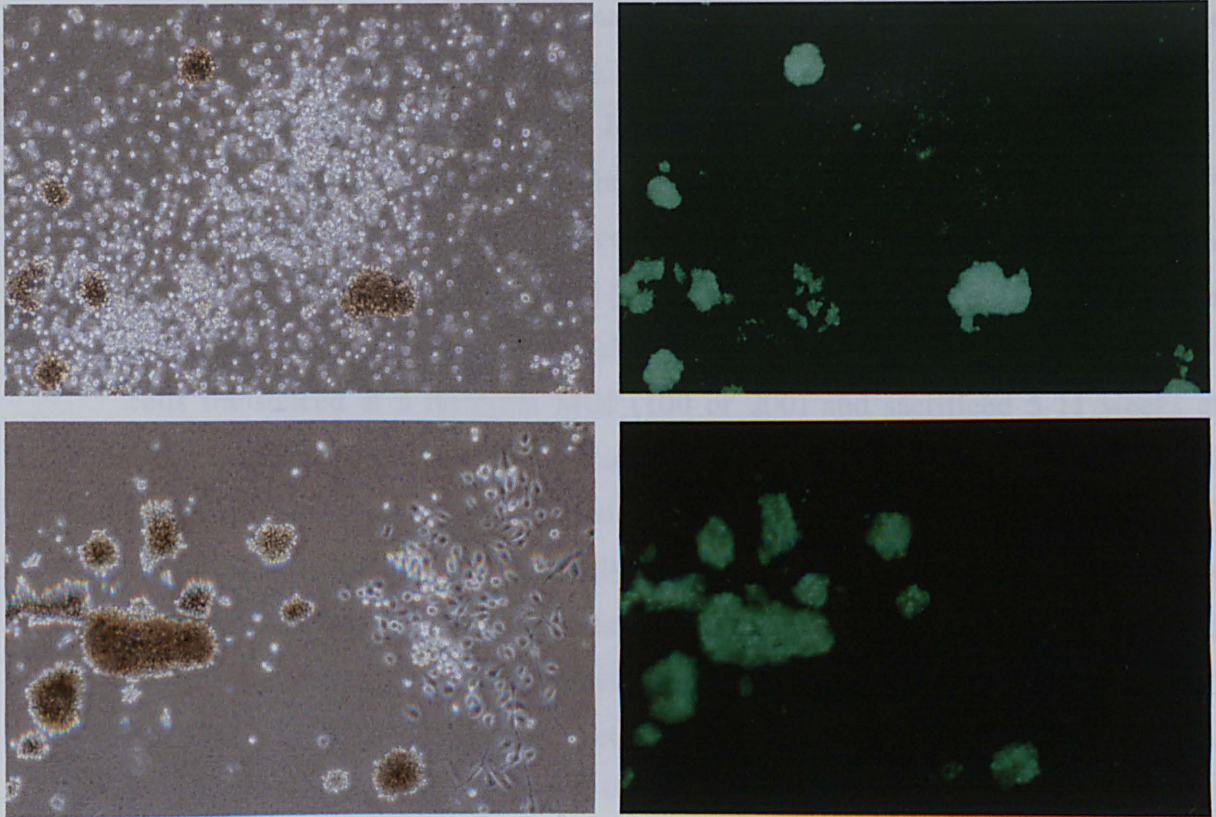


Figure 7.1. Expression of GFP (upper panels) and Δ LNGFR (lower panels) in liquid culture of human CD34⁺ hematopoietic stem/progenitor cells transduced with the G.W/P vector and analyzed 3 days after infection (left panels) or 10 days after induction of myelomonocytic or erythroblastic differentiation (center and right panels). Cells were analyzed by triple-immunofluorescence flow cytometry after staining with a TC-conjugated anti-LNGFR antibody and PE-conjugated antibodies against specific cell surface markers for undifferentiated progenitors (CD34 right panels), or myeloid (CD13, center panels) and erythroid (GpA, right panels) progeny. % values in the gated areas indicate cells double positive for each surface marker and either GFP (upper panels) or Δ LNGFR (lower panels) expression.

differentiating from transduced progenitors. Colonies were morphologically scored as BFU-E, CFU-GM or CFU-GEMM between 10 and 14 days after plating. No difference in colony-forming efficiency was found in control, mock-transduced *vs* G.W/P-transduced and immunoselected cells. In the representative experiment shown in Fig. 7.2, most BFU-E colonies (54 out of 57) scored strongly positive for GFP by fluorescence microscopy, while only rare CFU-GM colonies (2 out of 49) appeared weakly positive by the same assay.

Overall, these results show that transcription from the GATA-LTR is active at low level in a fraction of the CD34⁺ hematopoietic progenitors, strongly down-regulated during myelo-monocytic differentiation and activated and maintained at high levels in cells undergoing erythroid differentiation.



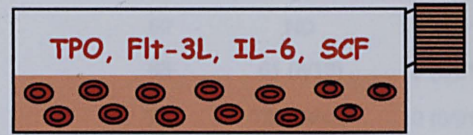
of human origin (CD45⁺) and 7.2% of human CD45⁺ cells expressed Δ LNQFR. Further staining with antibodies against the lineage markers CD19, CD13 and CD34 showed the presence of human lymphoid, myeloid and multi-lineage progenitor cells. LNQFR⁺ cells were found in a similar percentage in all tested lineages (Fig. 7.4). GFP expression was detected in < 1% of human cells recovered from BM of all but one of the six analyzed mice. In a single instance (cf. Table 7.1) GFP expression was detected in 3.7%, 3.0% and 3.6% of transduced CD45⁺, CD34⁺ and CD19⁺ cells, respectively, suggesting the presence in this animal of a high proportion of immature GATA-1-expressing cells (myeloid and lymphoid progenitors). Since human erythroid differentiation is controlled by a very marked up-regulation of GATA-1 expression in developing CD34⁺ cells, it is very unlikely that the Δ LNQFR-driven expression of the GATA-LTR in the erythroid lineage property of transduced SCID-repopulating

Figure 7.2. Erythroid-specific expression of GFP in methylcellulose colonies derived from CD34⁺ human hematopoietic progenitors transduced with the G.W/P lentiviral vector (see Fig. 5), and immunoselected for Δ LNQFR expression before plating. A,C, bright-field view of fully mature BFU-E and CFU-GM colonies 14 days after plating; B,D, green fluorescence view of the same fields (GFP-specific filters). Magnification: 10 X

7.2 Expression of the GATA-LTR in human SCID-repopulating cells.

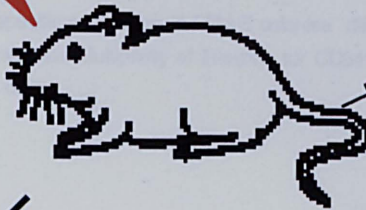
The lineage-specific expression of the GATA-LTR was tested in human hematopoietic stem/progenitor cells *in vivo* in the NOD/SCID mouse model as represented in the experimental design illustrated in Fig. 7.3. Briefly, cord blood CD34⁺ cells were transduced with the G.W/P vector, further modified by the addition of the HIV-1 cPPT sequence which was recently reported as essential for high efficiency of gene transfer into SCID-repopulating cells (SRCs) [Follenzi, 2000 #157]). A total of 10 mice in three different experiments were sub-lethally irradiated, inoculated with $1.5-3 \times 10^5$ CD34⁺ cells transduced at a different MOI (100 or 200) and sacrificed 5-11 weeks after transplantation (Fig. 7.3). Transduction efficiency of CD34⁺ cells ranged from 49 to 76% as assayed by Δ LNGFR expression 4 days after infection (Table 7.1). Engraftment of human cells ranged from 1.5 to 58% as indicated by the proportion of human CD45⁺ cells in mouse BM. Gene transfer efficiency in engrafted human cells ranged from 35 to 72% as assayed by Δ LNGFR expression (Table 7.1). A representative analysis of the BM of a NOD/SCID mouse that received a transplantation dose of 3×10^5 CD34⁺ cells transduced at a proportion of 76% is shown in Fig. 7.4. Here, 58% of the BM cells were of human origin (CD45⁺) and 72% of human CD45⁺ cells expressed Δ LNGFR. Further staining with antibodies against the lineage markers CD19, CD13 and CD34 showed the presence of human lymphoid, myeloid and undifferentiated progenitor cells. LNGFR⁺ cells were found in a similar percentage in all tested lineages (Fig. 7.4). GFP expression was detectable in < 1% of human cells recovered from BM of all but one of the ten analyzed mice. In a single mouse (#1.3 in Table 7.1) GFP expression was detected in 33%, 40% and 36% of transduced CD45⁺, CD34⁺ and CD19⁺ cells respectively, suggesting the presence in this animal of a high proportion of immature GATA-1-expressing multilineage and lymphoid progenitors. Since human erythroid differentiation occurs at a very low level *in vivo* in the NOD/SCID model, expression of the GATA-LTR in the erythroblastic progeny of transduced SRCs was analyzed by plating BM cells from transplanted and control NOD/SCID mice in clonal

CD34+ cells from
cord blood



Transduction with lentivector
(MOI 100-200)

300 Rad



5-11 wks

BM
CFC assay



Blood, BM
FACS analysis



Figure 7.3. Transplantation of transduced CD34+ CBCs in NOD/SCID mice.

Table 7.1. Transduction of SRCs with G.W/P vector

Group	Mouse	Weeks after transplantation	MOI	TE FACS (%)	Cell dose (10 ⁵)	human cell engraftment(% CD45 ⁺)	LNGFR ⁺ human cells(%)	GFP ⁺ BFU-E colonies (%)	GFP ⁺ CFU-GM colonies (%)
I	1.1	5	200	76	3	2.5	62	ND	ND
	1.2	5	200	76	3	3	63	61 (8/13)	0 (0/21)
	1.3	9	200	76	3	58	72	73 (29/40)	0 (0/91)
II	2.1	9	200	68	2	50.5	44	52 (16/31)	0 (0/63)
	2.2	9	200	68	2	6.5	64	ND	ND
	2.3	9	200	68	2	9.4	54	ND	ND
	2.4	9	200	68	2	23.7	43	46 (11/24)	0 (0/43)
III	3.1	11	100	49	1.5	5.7	47	54 (6/11)	0 (0/24)
	3.2	11	100	49	1.5	4	35	ND	ND
	3.3	11	100	49	1.5	1.5	45	ND	ND

G.W/P lentiviral vector confers erythroid-specific expression in BFU-E colonies differentiating from transduced SRCs. The result from three different experiments are shown. MOI indicates Multiplicity of infection for CD34⁺ cells. TE indicates transduction efficiency assessed by FACS 4 days after transduction. ND, not done.

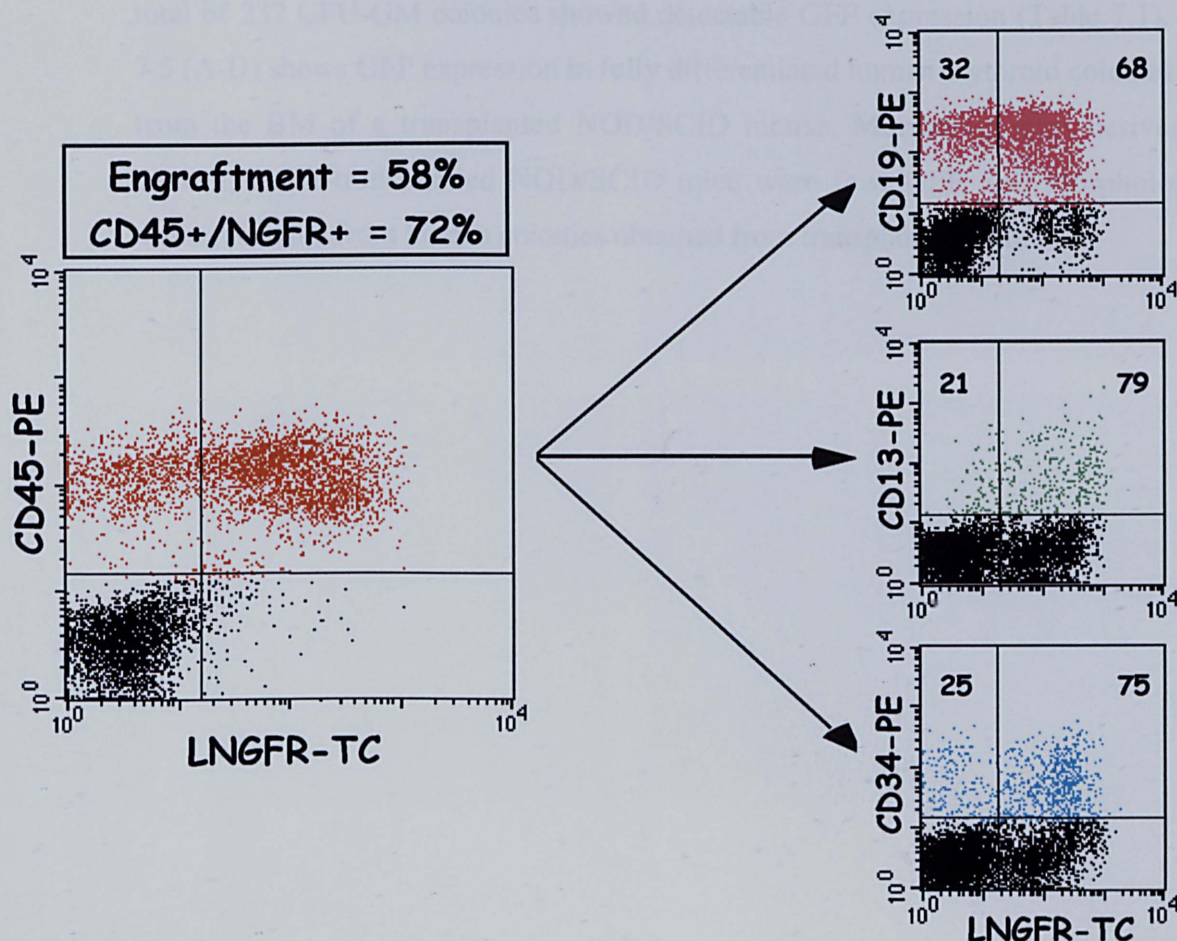
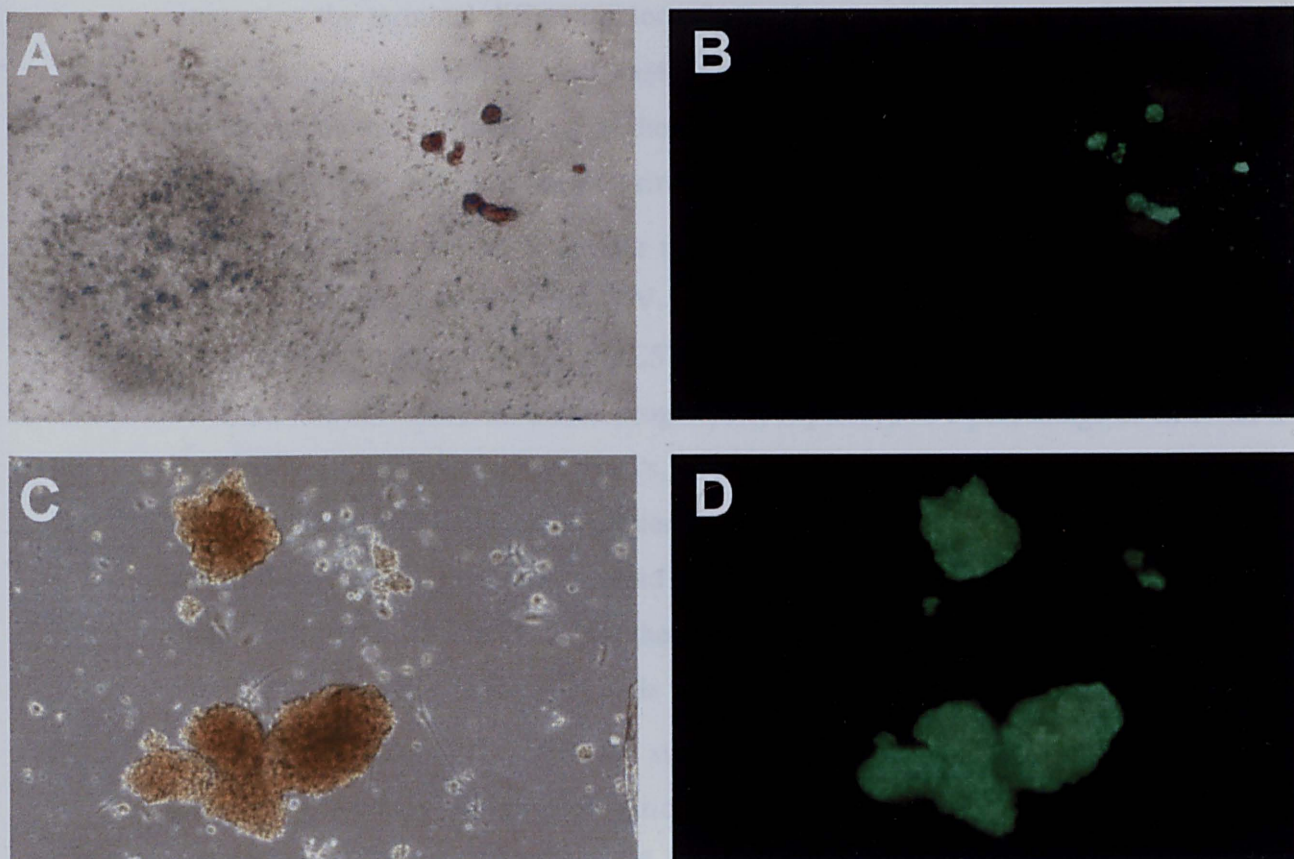


Figure 7.4. Multilineage reconstitution of the bone marrow of a NOD/SCID mouse transplanted with cord blood-derived human CD34⁺ hematopoietic stem/progenitor cells transduced with the G.W/P lentiviral vector (see Fig. 5) at a MOI of 200. Transduction efficiency in the transplanted cells was measured by FACS analysis of Δ LNGFR expression 4 days after infection (upper left panel). Cells were transplanted in 10 different NOD-SCID mice (see Table 1 for complete data). Engraftment was analyzed by FACS analysis of the human-specific CD45 marker in bone marrow cells 9 weeks after transplantation (lower left panel). Transduction efficiency in the total engrafted cell population (CD45⁺, lower left panel), and in the progenitor (CD34⁺), B lymphoid (CD19⁺) and myeloid (CD13⁺) sub-populations (right panels) were analyzed by Δ LNGFR expression. The percentage of LNGFR-positive and -negative cells within each cell sub-population is indicated in the appropriate quadrants.

cultures under conditions that preferentially support outgrowth of human progenitors (Cashman and Eaves, 1999). Following 11-14 days after plating, single colonies were counted and scored for GFP expression under an inverted fluorescence microscope. Overall, 70 out of 119 BFU-E colonies were GFP⁺ (average: 59%), while none out of a total of 232 CFU-GM colonies showed detectable GFP expression (Table 7.1). Figure 7.5 (A-D) shows GFP expression in fully differentiated human erythroid colonies grown from the BM of a transplanted NOD/SCID mouse. Murine colonies derived from control, mock-transplanted NOD/SCID mice were few, GFP⁻ and morphologically distinguishable from human colonies obtained from transplanted animals.

7.3 Long-term erythroid-specific expression of the GATA-LTR in murine repopulating SRCs

To test the lineage-specific expression of the GATA-LTR in a transplantation model



expression of enriched donor cells in BML-TEN/19%GFP cells ranged from 1.2 to 100%. These results indicate that expression of the GATA-LTR is restricted to the fully differentiated erythroid progeny of transduced mouse-repopulating stem cells in

Figure 7.5. Erythroid-specific expression of GFP in methylcellulose colonies derived from human NOD-SCID mouse-repopulating cells (SRCs) transduced with the G.W/P lentiviral vector (see Fig. 13) and analyzed *ex vivo* 9 weeks after transplantation. A,C, bright-field view of fully mature BFU-E and CFU-GM colonies 14 days after plating; B,D, green fluorescence view of the same fields (GFP-specific filters). Magnification: 4X in A, B, 10 X in C, D.

7.3 Long-term erythroid-specific expression of the GATA-LTR in murine repopulating HSCs.

To test the lineage-specific expression of the GATA-LTR in a transplantation model supporting the terminal differentiation of all progeny derived from transduced HSCs, we performed bone marrow transplantation of transduced cells in congenic mice using inbred strains that differed only at the CD45 molecule by two known alleles, CD45.1 and CD45.2. We followed the experimental plan illustrated in Fig. 7.6. BM cells (5×10^6) from C57Bl/6 (CD45.2⁺) donor mice were transduced with the G vector, further modified by the addition of the HIV-1 cPPT and WPRE sequences (MOI: 200), and transplanted into lethally-irradiated C57Bl/6-Ly-5.1 (CD45.1⁺) recipient mice (Fig. 7.6). Transduction efficiency of murine progenitors, expressed as a percentage of GFP⁺ BFU-E colonies in methylcellulose assays, averaged 75%. After 9 to 11 weeks following transplantation, BM cells from recipient animals were analyzed for expression of GFP, and erythroid (TER-119) and myeloid (Gr-1) lineage-specific markers. Engraftment of donor cells in the BM, indicated by the proportion of CD45.2⁺/total CD45⁺ cells, ranged from 26 to 94% (Fig. 7.7). GFP⁺ cells were detected at a significant level (3.0 to 39%), only in the erythroblastic, TER119⁺ sub-population of BM cells (Fig. 7.8, upper right panel). Conversely, in myeloblastic Gr-1⁺ cells GFP expression was virtually undetectable (0.6 to 1.3%) (Fig. 7.8, lower right panel). After normalization for the proportion of engrafted donor cells in BM, TER119⁺/GFP⁺ cells ranged from 3.2 to 96%. These results indicate that expression of the GATA-LTR is restricted to the fully differentiated erythroblastic progeny of transduced mouse-repopulating stem cells *in vivo*.

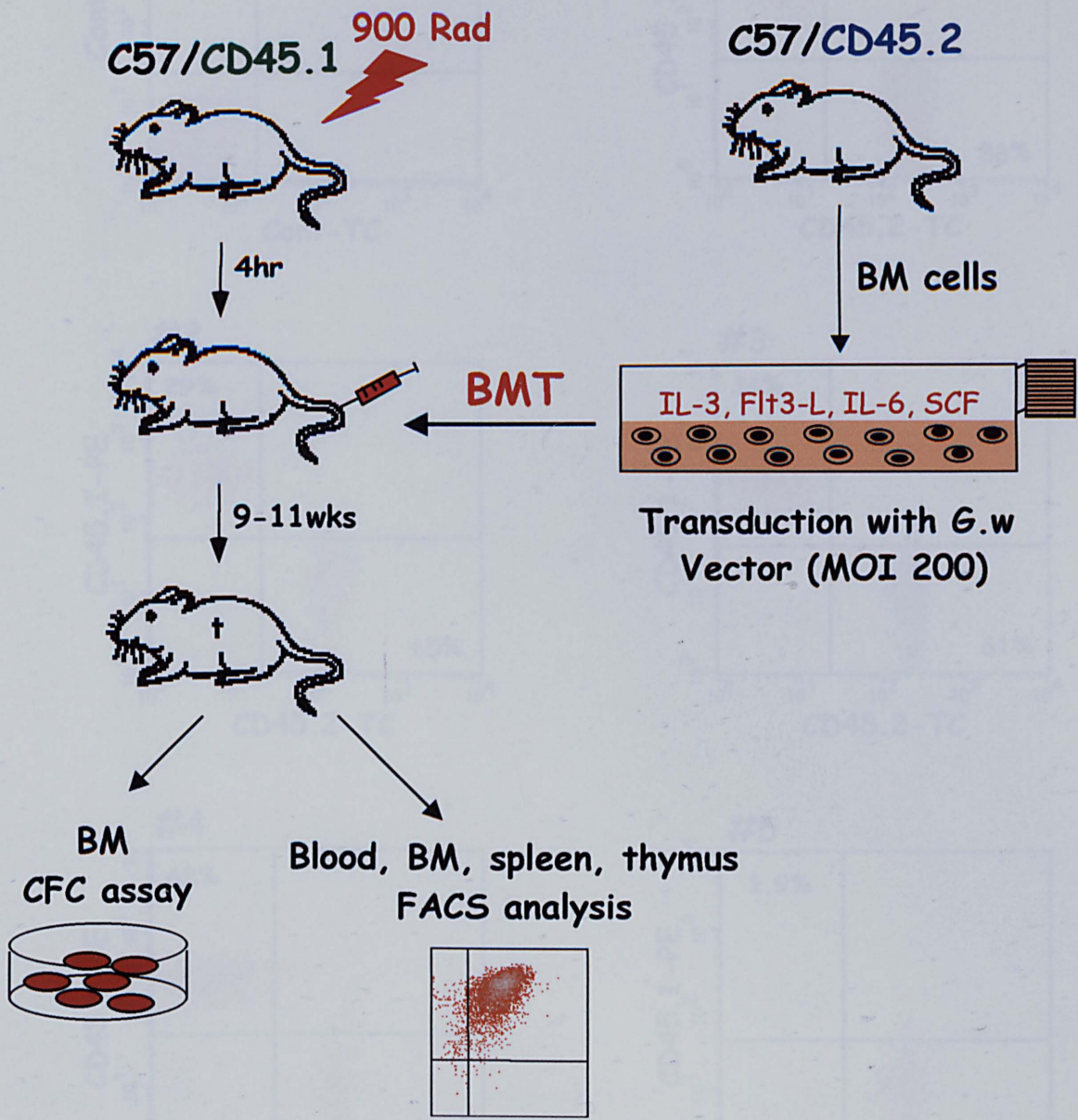


Figure 7.6. Transplantation of murine BM-transduced cells.

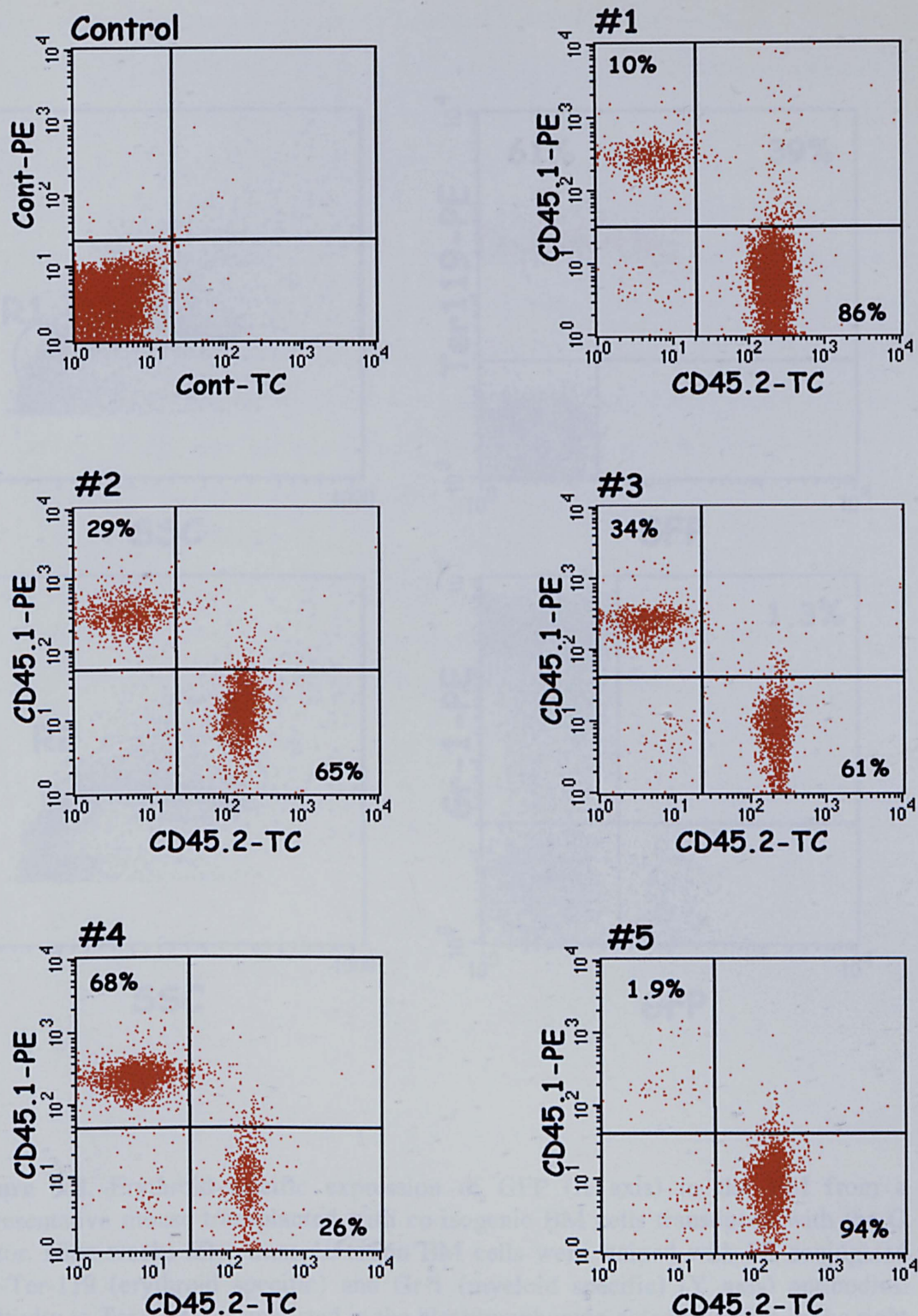


Figure 7.7. Level of chimerism expressed as the proportion of CD45.2⁺/total CD45.1⁺ cells in the BM from 5 mice transplanted with co-isogenic BM cells transduced with the G vector. After 9 to 36 weeks following transplantation BM cells were stained with PE-conjugated anti-CD45.1 (Y axis) and with FITC-conjugated anti-CD45.2 (X axis) antibodies. The percentage of CD45.1⁺ and CD45.2⁺ cells is indicated in the appropriate quadrants. In the first panel is reported the isotype control for the PE and FITC expression of the BM cells of one representative mouse (#2).

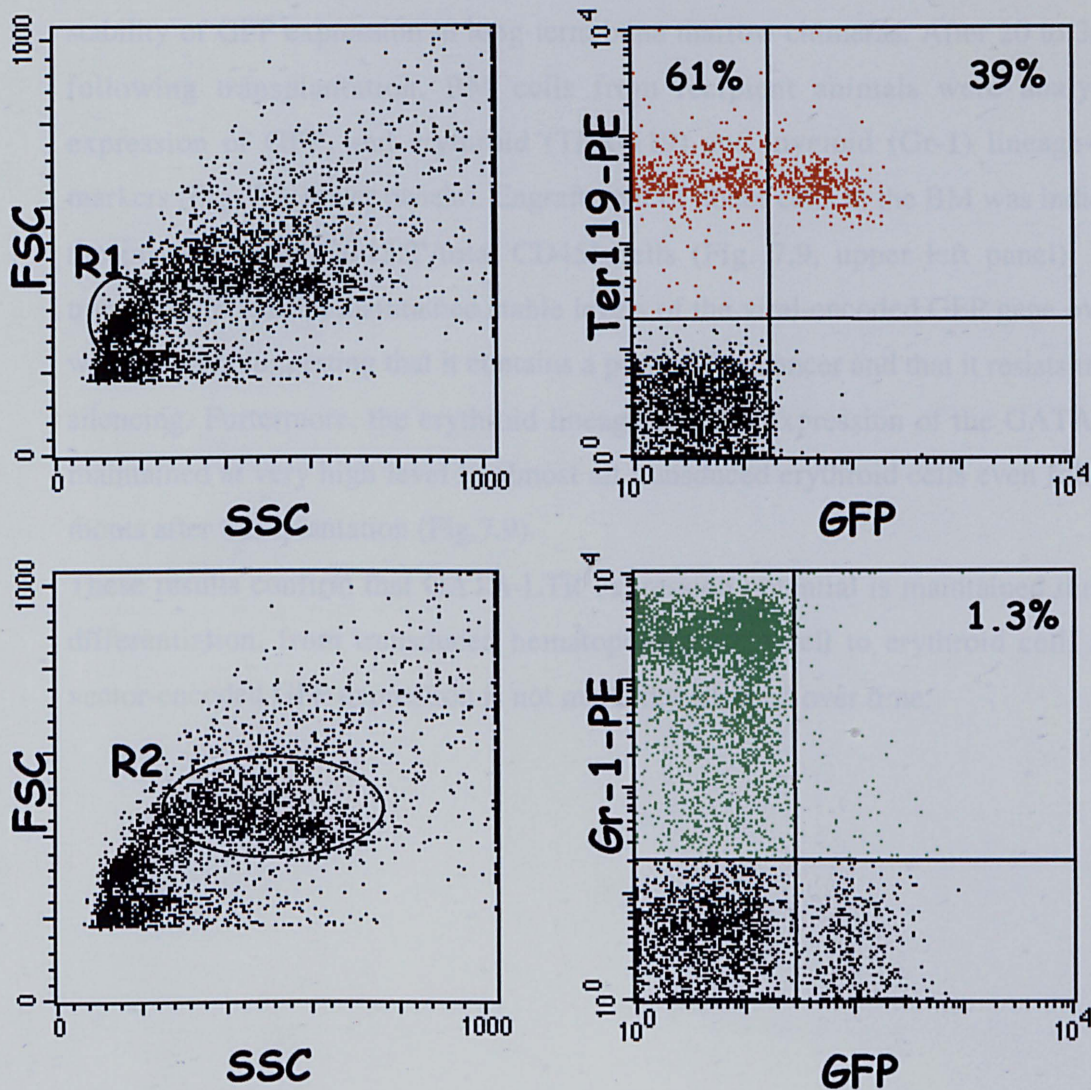


Figure 7.8. Erythroid-specific expression of GFP (X axis) in the BM from a representative mouse transplanted with co-isogenic BM cells transduced with the G vector. Nine weeks after transplantation BM cells were stained with PE-conjugated anti-Ter-119 (erythroid specific) and Gr-1 (myeloid specific) (Y axis) antibodies. Positivity to Ter-119 was analyzed in the blast/lymphocyte-gated cells (R1 in the right upper panel), and positivity to Gr-1 was analyzed in the granulo/monocyte-gated cells (R2 in the right lower panel). The percentage of GFP-positive and -negative cells within each cell sub-population is indicated in the appropriate quadrants (left panels). The degree of chimerism in this representative animal, expressed as the proportion of CD45.2⁺/total CD45⁺ cells, was 67.5%. SSC: side scatter; FCS: forward scatter.

One major limitation to a genetic approach of therapy is the possibility of transcriptional silencing of the integrated vectors over time (Challita, 1994). Therefore, we studied the stability of GFP expression in long-term bone marrow chimeras. After 20 to 36 weeks following transplantation, BM cells from recipient animals were analyzed for expression of GFP, and erythroid (TER-119) and myeloid (Gr-1) lineage-specific markers (Fig. 7.9, lower panels). Engraftment of donor cells in the BM was indicated by the proportion of CD45.2⁺/total CD45⁺ cells (Fig. 7.9, upper left panel). Primary transplant recipients maintained stable levels of the viral-encoded GFP gene over a 36-week period, suggesting that it contains a powerful enhancer and that it resists transgene silencing. Furthermore, the erythroid lineage-specific expression of the GATA-LTR is maintained at very high level in almost all transduced erythroid cells even following 9 months after transplantation (Fig. 7.9).

These results confirm that GATA-LTR expression potential is maintained throughout differentiation, from transduced hematopoietic stem cell to erythroid cell, and that vector-encoded GFP expression is not markedly silenced over time.

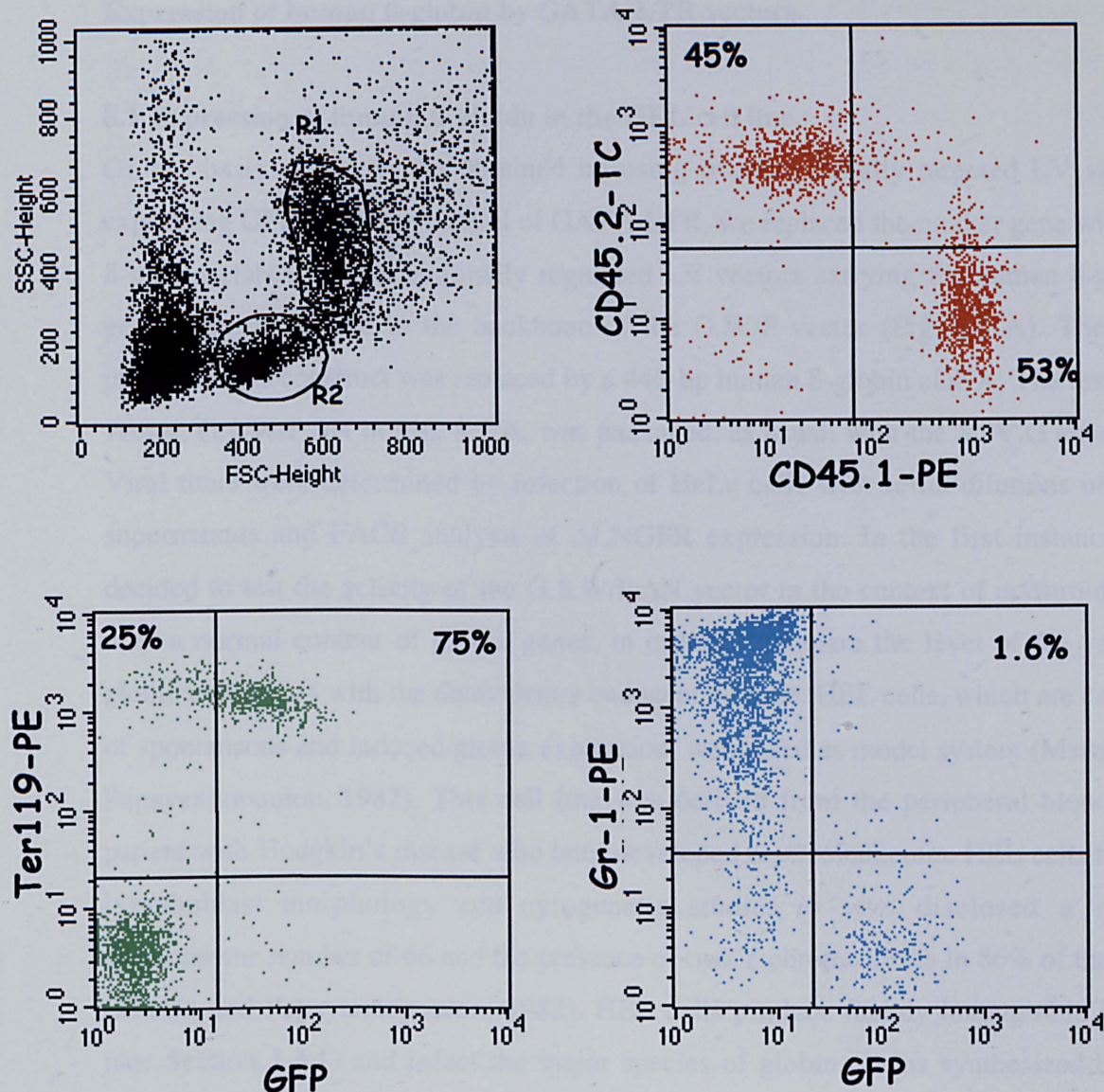


Figure 7.9. Level of chimerism and long-term erythroid-specific expression of GFP (X axis) in the BM from a representative mouse transplanted with co-isogenic BM cells transduced with the G vector. Nine months (36 weeks) after transplantation BM cells were stained with PE-conjugated anti-Ter-119 (erythroid specific), Gr-1 (myeloid specific), CD45.1 (Y axis) and TC-conjugated CD45.1 antibodies. Positivity to Ter-119 was analyzed in the blast/lymphocyte-gated cells (R1 in the right upper panel), and positivity to Gr-1 and CD45 was analyzed in the granulo/monocyte-gated cells (R2 in the right lower panel). The percentage of GFP-positive and -negative cells within each cell sub-population is indicated in the appropriate quadrants (left panels).

Chapter 8

Expression of human β -globin by GATA-LTR vectors.

8.1 Expression of human β -globin in the HEL cell line.

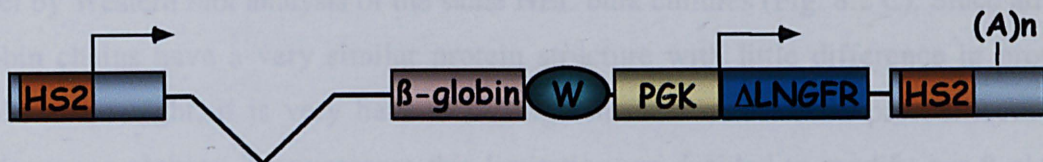
On the basis of the results obtained by using transcriptionally targeted LV vectors expressing GFP under the control of GATA-LTR, we replaced the marker gene with the β -globin cDNA. Transcriptionally regulated LV vectors carrying the human β -globin gene were developed in the backbone of the G.W/P vector (Fig. 6.3 A). The GFP protein in this construct was replaced by a 440-bp human β -globin cDNA. The resulting vector, G. β .W/P. Δ N in Fig. 8.1 A, was packaged, as usual, with the VSV.G envelope. Viral titers were determined by infection of HeLa cells with serial dilutions of viral supernatants and FACS analysis of Δ LNGFR expression. In the first instance, we decided to test the activity of the G. β .W/P. Δ N vector in the context of erythroid cells with a normal content of globin genes, in order to compare the level of the viral β -globin expression with the equivalent γ endogenous gene. HEL cells, which are capable of spontaneous and induced globin expression, were used as model system (Martin and Papayannopoulou, 1982). This cell line was derived from the peripheral blood of a patient with Hodgkin's disease who later developed erythroleukemia. HEL cells have a lymphoblast morphology and cytogenetic studies *in vivo* disclosed a modal chromosome number of 66 and the presence of two Y chromosomes in 86% of the cells (Martin and Papayannopoulou, 1982). HEL cells produce mainly hemoglobin Bart's (see Section 1.3.1) and infact the major species of globin chains synthesized by the these cells were γ chains ($G\gamma$, $A\gamma$, and their acetylated forms). The same pattern of globin chain synthesis was also observed in hemin-induced HEL cells. Quantitative studies indicated a ten-fold increase in globin synthesis after hemin induction. This induction of HEL cells by hemin is associated with quantitative rather than qualitative changes in globin genes, though the same group, more recently, reported that hemin induction cosinstantly increases the proportion of α -chains (Papayannopoulou *et al.*, 1987). Recently, the mechanism of action of hemin has been elucidated. It has been reported that hemin overcomes the inibition of translation due to the phosporylation of the α subunit of eIF-2 operated by HRI kinase. HRI kinase has two binding sites for heme and hemin binding to these sites inhibits HRI function, enhancing the level of globin mRNA translation.

In order to test the biological activity of the G.β.W/P.ΔN vector, HEL cells were transduced at an MOI of 5 using the spinoculation protocol described in Materials and Methods. Transduced cells were maintained as bulk culture and three days after infection, transduction efficiency was evaluated by FACS analysis of ΔLNGFR expression (Fig. 8.1 B). Southern blot analysis of genomic DNA, digested with *Afl*III and hybridized to a WPRE probe, showed stable integration of intact provirus with the expected size of 4.5 kb (Fig. 8.2 A). End-fragment analysis of genomic DNA with *Xba*I, which cuts once inside the vector, showed the polyclonal nature of the HEL bulk culture (Fig. 8.2 A).

The transcriptional activity of the G.β.W/P.ΔN vector was characterized by Northern blot analysis of transduced HEL cells, with or without induction of differentiation in the presence of 50 μM hemin (Fig. 8.2 B). Four days after induction of differentiation, poly-A⁺ RNA was extracted from differentiated and non-differentiated HEL cells and analyzed by Northern blotting after hybridization with a β-globin probe (Fig. 8.2 B, upper panel). Since the nucleotide sequences of α-, γ- and β-globins are very similar, using β-globin as a probe is possible to detect the signal relative to the viral β-globin (around 3.6 kb, Fig. 8.2 B) and the band relative to the endogenous cellular α- and γ-globins (around 0.7 kb, Fig. 8.2 B). This allows a comparison in the level of viral β-globin expression with that of endogenous globins by phosphorimaging of Northern blots after normalization to GAPDH mRNA content (Fig. 8.2 B, bottom panel). In undifferentiated cells the level of viral β-globin transcript was approximately 12-fold higher than the endogenous γ gene (Fig. 8.2 B, second lane), while in hemin-induced cells the viral signal was 230% of the endogenous α/γ genes (Fig. 8.2 B, fourth lane).

A

RRL.sin-40.β-globin.Wpre.PGK.ΔN (G.β.W/P.ΔN)



B

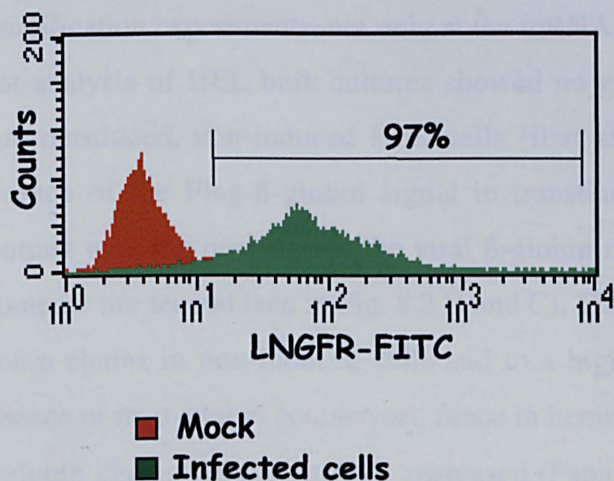


Figure 8.1. (A) Schematic map of the RRL.GATA.β-globin.W.PGK.ΔN (G.β.W/P.ΔN) vector in its proviral form. The FLAG-modified human β-globin cDNA is inserted in place of the GFP cDNA in the G.W/P vector described in Fig 3.13 A. (B) Analysis of LNGFR expression in HEL cells transduced with the G.β.W/P.ΔN vector. HEL cells were infected with vector at a MOI of 5, grown as bulk cultures and analyzed for LNGFR expression by single-channel cytofluorimetry after staining with a FITC-conjugated anti-LNGFR antibody (X axis). Expression profiles are indicated by red (mock infected cells) or green (infected cells) peaks respectively. Percentage of LNGFR expressing cells is indicated within the panel.

As expected, no viral β -globin signals were detected in non-transduced HEL cells (first and third lanes in Fig. 8.2 B).

Human β -globin expression, driven by GATA-LTR, was evaluated also at the protein level by Western blot analysis of the same HEL bulk cultures (Fig. 8.2 C). Since all the globin chains have a very similar protein structure with little difference in protein molecular weight, it is very hard to distinguish the viral derived protein from the endogenous globins. To overcome this limitation we decided to modify our β -globin cDNA by adding an identification tag. For this reason, a FLAG epitope was cloned in frame at the 5' end of the β -globin cDNA. This epitope allowed detection of viral β -globin by a specific anti-FLAG antibody. Furthermore, due to the enhancement of the molecular weight of the Flag- β -globin, this allowed it to be distinguished from the endogenous band of globins also when we use a polyclonal antibody that recognize all the globin chains. This modification in the viral β -globin allowed us to perform quantification experiments, not only at the mRNA, but also at the protein level. Western blot analysis of HEL bulk cultures showed no globin signals in both transduced and non-transduced, non-induced HEL cells (first and second lanes in Fig. 8.2 C). The absence of the Flag- β -globin signal in transduced, non-induced HEL cells was in contrast with the presence of the viral β -globin mRNA in the same HEL bulk culture (compare the second lane in Fig. 8.2 B and C). This is probably due to the absence of α -globin chains in non-induced cells and to a high rate of β -globin degradation in the absence of its α -globin counterpart. Since in hemin-induced HEL cells the proportion of α -globin chains are consistently increased (Papayannopoulou et al., 1987), this could enhance the stability of the viral β -globin by association with the induced α -globin in hemoglobin tetramers. Western blot analysis of HEL bulk cultures also showed that Flag- β -globin accumulated at a level of 42% with respect to the endogenous human globin chains (fourth lane in Fig. 8.2 C), while, as expected, no Flag- β -globin signal was detected in non-transduced, hemin-induced HEL cells (third lane in Fig. 8.2 C). The specificity of the Flag- β -globin signal was confirmed by Western blot analysis of hemin induced HEL cells using a monoclonal antibody specific for the FLAG epitope (Fig. 8.2C).

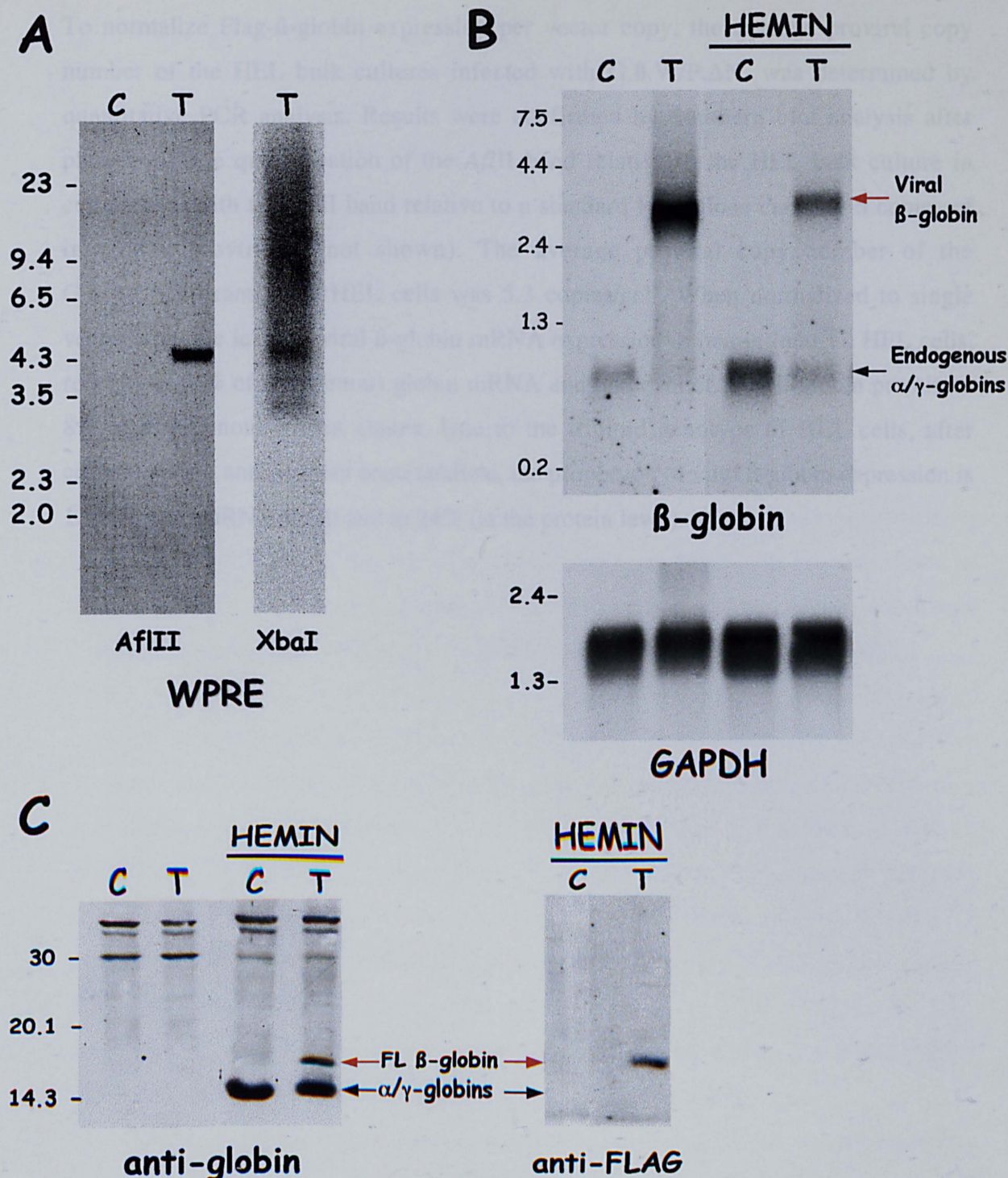


Figure 8.2. (A) Southern blot analysis of genomic DNA extracted from mock-transduced (C) or G.B.W/P.ΔN-transduced (T) HEL cells, digested with *Afl*III or with *Xba*I and hybridised with a WPRE probe. (B) Northern blot analysis of poly-A⁺ RNA extracted from the same HEL cells with or without induction of differentiation with hemin, hybridised with a β-globin (upper panel) or a GAPDH (lower panel) probe. Viral and endogenous globin bands are indicated on the right. (C) Western blot analysis of proteins extracted from the same HEL cells and bound with a polyclonal anti-globin or monoclonal anti-FLAG antibody. Bands relative to the Flag-β-globin and to the α/γ globins were indicated on the right. Molecular weights in kilobase (A and B) and in kilodalton (C) are indicated on the left.

To normalize Flag- β -globin expression per vector copy, the average proviral copy number of the HEL bulk cultures infected with G. β .W/P. Δ N, was determined by quantitative PCR analysis. Results were confirmed by Southern blot analysis after phosphorimage quantification of the *Afl*III band relative to the HEL bulk culture in comparison with the *Afl*III band relative to a standard HEL clone carrying 5 copies of integrated proviruses (not shown). The average proviral copy number of the G. β .W/P. Δ N-transduced HEL cells was 5.3 copies/cell. When normalized to single vector copy the level of viral β -globin mRNA expression in hemin-induced HEL cells, reduces to 43% of endogenous globin mRNA and the level of Flag- β -globin protein to 8% of endogenous globin chains. Due to the triploid genotype of HEL cells, after correction to α and γ -globin content/allele, the proportion of viral β -globin expression is 126% (at the mRNA level) and to 24% (at the protein level).

8.2 Expression of human β -globin in the MEL cell line.

Similar results were obtained when we use the murine erythroleukemia (MEL) cell line as an erythroid expression system. MEL cells are erythroid progenitor cells derived from the spleens of susceptible mice infected with the Friend virus complex. These virally transformed cells are arrested at the proerythroblast stage of development and can be maintained in culture indefinitely (Singer et al., 1974). Most MEL cell lines show a low (<1%) level of spontaneous erythroid differentiation, however, upon treatment with various chemical agents, MEL cells can be induced to undergo erythroid differentiation at much higher levels (30-100%). Among the most potent inducers of differentiation are polar-planar compounds (such as dimethyl sulfoxide, hexamethylene bisacetamide, sodium butyrate and retinoic acid). Erythroid differentiation of MEL cells in culture recapitulates many aspects of the erythroid terminal differentiation program, including hemoglobin synthesis and growth arrest. For this reason, MEL cells are considered an excellent model system for studying changes in chromatin structure and coordinate globin gene expression associated with differentiation (Fraser and Curtis, 1987).

To test the activity of the G. β .W/P. Δ N vector (Fig. 8.3 A), MEL cells were transduced with the viral supernatant at an MOI of 10, using the spinoculation protocol (see Materials and Methods). Transduced cells were maintained as bulk cultures and three days after infection, transduction efficiency was evaluated by FACS analysis of Δ NGFR expression (Fig. 8.3 A). Cells in the bulk culture were almost all transduced (99%) and vector DNA stability was determined by genomic Southern blot analysis (Fig 8.3 B). Genomic DNA digestion with *A*fIII and hybridization with a WPRE probe, showed stable integration of intact provirus with the expected size of 4.5 kb (Fig. 8.3 B). End-fragment analysis of genomic DNA with *X*baI, which cuts once inside the vector, showed the polyclonal nature of the HEL bulk culture (Fig. 8.3 B).

The transcriptional activity of the G. β .W/P. Δ N vector in MEL cells was characterized by Northern blot analysis, with or without induction of differentiation in the presence of 2% DMSO, after hybridization to a β -globin probe (Fig. 8.4 A). Hybridization with a β -globin probe allowed a comparison in the level of viral β -globin expression with the endogenous murine α and β -globins in DMSO-induced MEL cells transduced with the G. β .W/P. Δ N vector. In contrast to HEL cells, no endogenous β -globin expression was detected in non-induced MEL cells (compare the first two lanes in Fig. 8.2 B and in Fig. 8.4 A). However, after transduced MEL cells were induced with DMSO, the viral β -globin transcripts accumulated at a level of 70% with respect to the level of endogenous

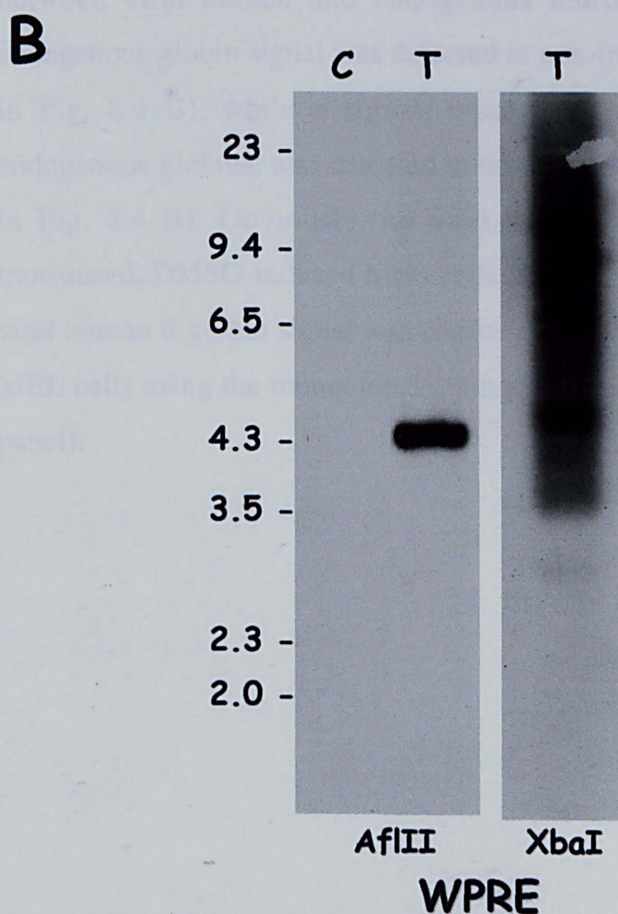
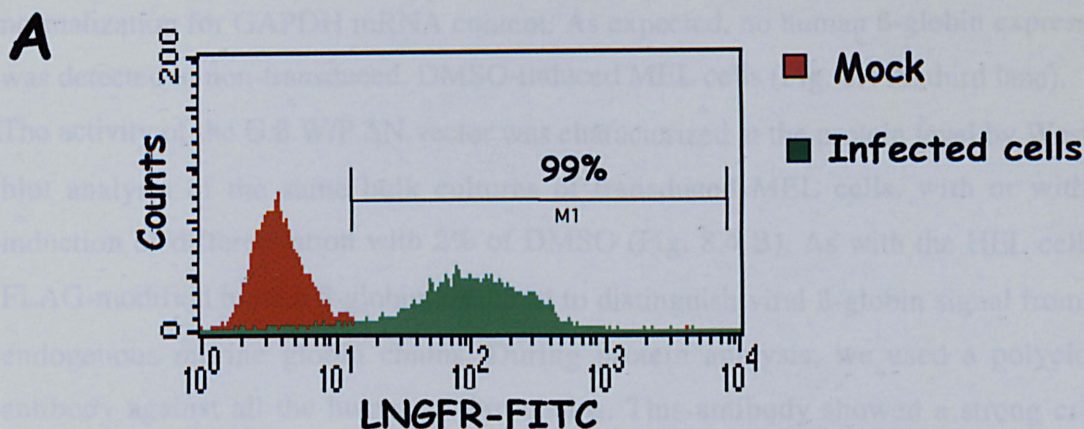


Figure 8.3. (A) Analysis of LNGFR expression in MEL cells transduced with the G.B.W/P. Δ N vector. MEL cells were infected with the vector at a MOI of 10, grown as bulk cultures and analyzed for LNGFR expression by single-channel fluorescence cytometry after staining with a FITC-conjugated anti-LNGFR antibody (X axis). Expression profiles are indicated by red (mock infected cells) or green (infected cells) peaks, respectively. The percentage of LNGFR expressing cells is shown within the panel. (B) Southern blot analysis of genomic DNA extracted from mock-transduced (C) or G.B.W/P. Δ N-transduced MEL cells, digested with *Afl*III or with *Xba*I and hybridized with a WPRE probe.

transcripts (Fig. 8.4 A, fourth lane), as quantified by phosphorimaging after normalization for GAPDH mRNA content. As expected, no human β -globin expression was detected in non-transduced, DMSO-induced MEL cells (Fig. 8.4 A, third lane). The activity of the G. β .W/P. Δ N vector was characterized at the protein level by Western blot analysis of the same bulk cultures of transduced MEL cells, with or without induction of differentiation with 2% of DMSO (Fig. 8.4 B). As with the HEL cells, a FLAG-modified human β -globin was used to distinguish viral β -globin signal from the endogenous murine globin chains. During protein analysis, we used a polyclonal antibody against all the human globin chains. This antibody showed a strong cross-reactivity with murine globin chains and allowed us to perform quantitative experiments between viral human and endogenous murine globin proteins. As expected, no endogenous globin signal was detected in non-induced MEL cells (first and second lane in Fig. 8.4 B), while a signal, representing a level of 30% with respect to the endogenous globins, was detected in transduced, DMSO-induced MEL cells (third lane in Fig. 8.4 B). Obviously, no viral human β -globin signal was detected in non-transduced, DMSO-induced MEL cells (fourth lane in Fig. 8.4 B). The specificity of the viral human β -globin signal was confirmed by Western blot analysis of DMSO-induced MEL cells using the monoclonal antibody specific for the FLAG epitope (8.4 B, lower panel).

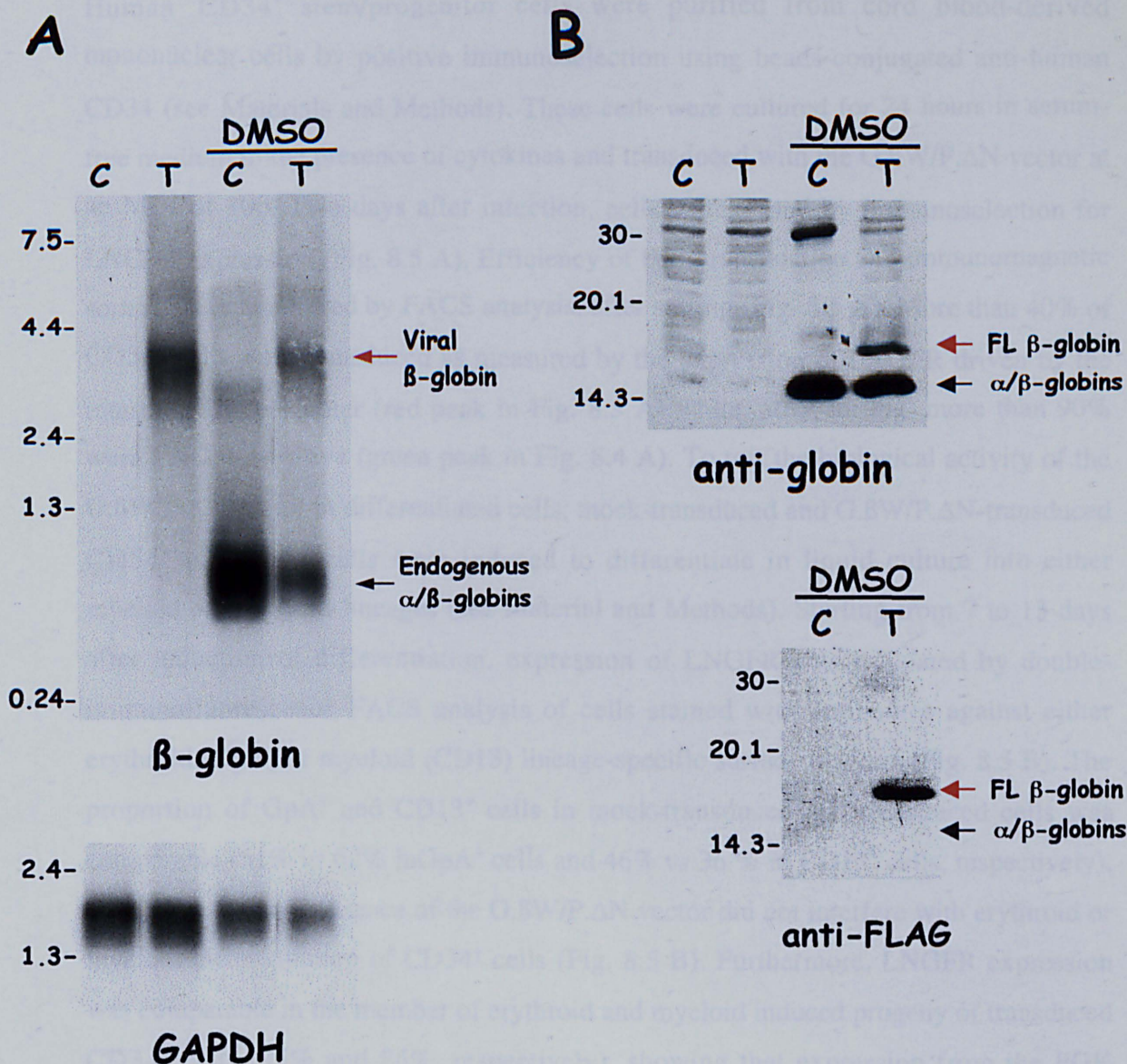


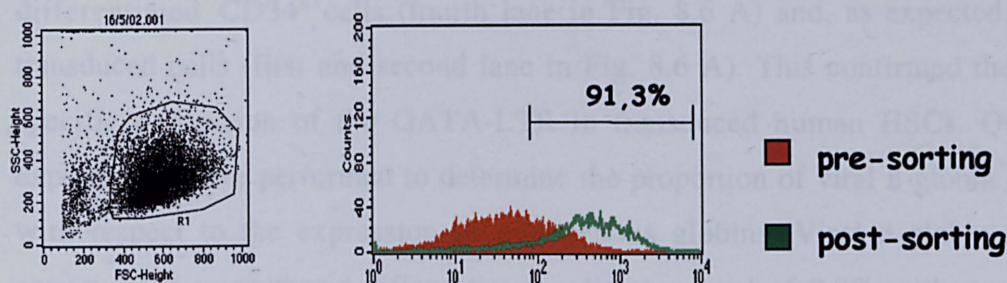
Figure 8.4. (A) Northern blot analysis of poly-A⁺ RNA extracted from mock-transduced (C) and G.B.W/P.ΔN-transduced MEL cells (T), with or without induction of differentiation with 2% DMSO, hybridised with β-globin (upper panel) and GAPDH (lower panel) probes. Viral and endogenous globin bands are indicated on the right. (B) Western blot analysis of proteins extracted from the same MEL cells and revealed using a polyclonal anti-globin or monoclonal anti-FLAG antibody. Bands relative to the Flag-β-globin and to the endogenous α/β globins are indicated on the right. Molecular weights in kilobase (A) and in kilodalton (B) are indicated on the left.

8.3 Expression of human β -globin in CD34⁺ cells.

Human CD34⁺ stem/progenitor cells were purified from cord blood-derived mononuclear cells by positive immunoselection using beads-conjugated anti-human CD34 (see Materials and Methods). These cells were cultured for 24 hours in serum-free medium in the presence of cytokines and transduced with the G. β W/P. Δ N vector at an MOI of 100. Two days after infection, cells were sorted by immunoselection for LNGFR expression (Fig. 8.5 A). Efficiency of both transduction and immunomagnetic sorting were monitored by FACS analysis, after sorting (Fig. 8.5 A). More than 40% of CD34⁺ cells were transduced as measured by the expression of LNGFR driven by the internal PGK promoter (red peak in Fig. 8.5 A) while, after sorting, more than 90% were LNGFR positive (green peak in Fig. 8.4 A). To test the biological activity of the G. β W/P. Δ N vector in differentiated cells, mock-transduced and G. β W/P. Δ N-transduced CD34⁺ progenitor cells were induced to differentiate in liquid culture into either myeloid or erythroid lineages (see Material and Methods). Starting from 7 to 13 days after induction of differentiation, expression of LNGFR was evaluated by double-immunofluorescence FACS analysis of cells stained with antibodies against either erythroid (GpA) or myeloid (CD13) lineage-specific surface markers (Fig. 8.5 B). The proportion of GpA⁺ and CD13⁺ cells in mock-transduced and transduced cells was comparable (51% vs 62% in GpA⁺ cells and 46% vs 36 % in CD13⁺ cells, respectively), indicating that the presence of the G. β W/P. Δ N vector did not interfere with erythroid or myeloid differentiation of CD34⁺ cells (Fig. 8.5 B). Furthermore, LNGFR expression was comparable in the member of erythroid and myeloid induced progeny of transduced CD34⁺ cells (95% and 86%, respectively), showing that expression from the PGK promoter is maintained at the same level under both differentiative conditions (Fig. 8.5 B).

To test the efficiency of the GATA-LTR to drive expression of the β -globin gene, both erythroid and myeloid-differentiated progeny of mock-transduced and transduced CD34⁺ cells were collected thirteen days after induction of differentiation and analyzed by Northern and Western blotting (Fig. 8.6). Total RNA extracted from differentiated CD34⁺ cells were analyzed by Northern blot after hybridization with a β -globin or a GAPDH probe (Fig. 8.6 A). The results show a strong activation of endogenous globin gene expression during erythroid differentiation, while no globin signals were detected in myeloid-differentiated CD34⁺ cells (Fig. 8.6 A, upper panel). The viral β -globin transcript was detected only in transduced, erythroid-differentiated CD34⁺ cells (third

A



B

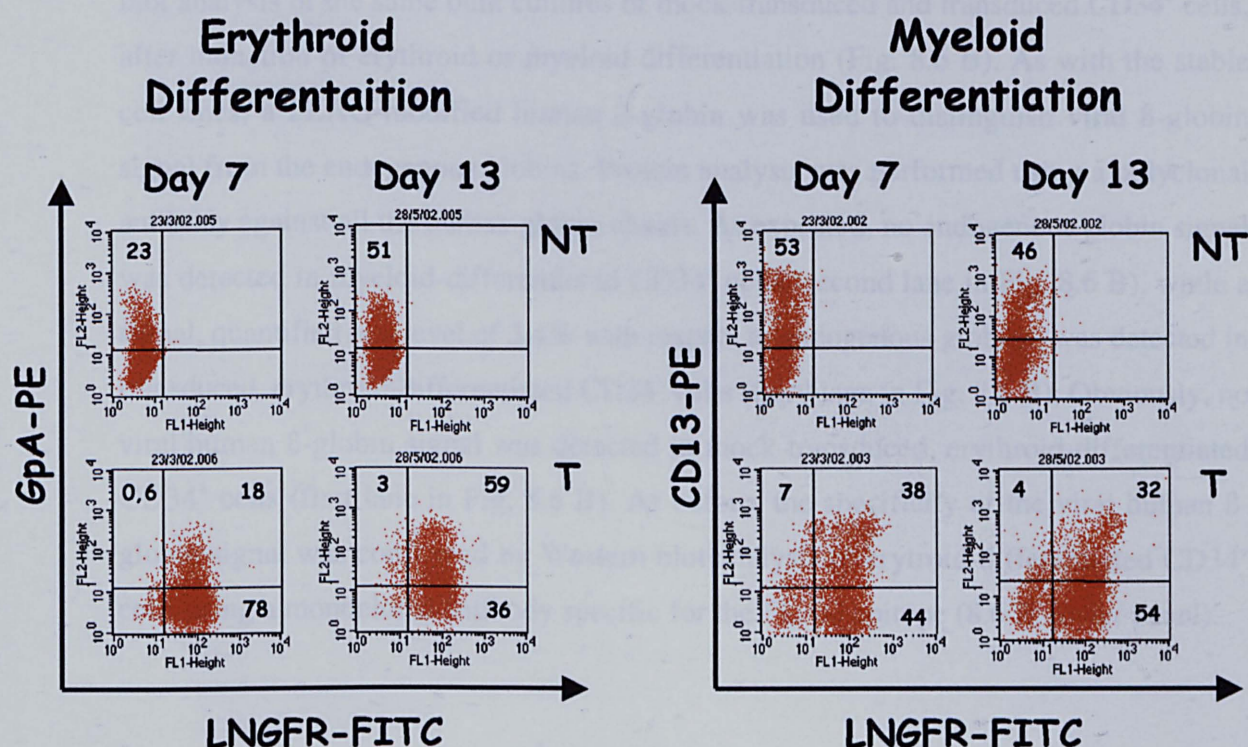


Figure 8.5. (A) FACS analysis of LNGFR expression in liquid cultures of human CD34⁺ hematopoietic stem/progenitor cells transduced with the G.BW/P.ΔN vector and analyzed 2 days after infection, before (red peak) and after immunomagnetic sorting (green peak) of LNGFR-expressing cells. (B) FACS analysis of the mock-transduced (NT) and G.BW/P.ΔN-transduced (T) CD34⁺ cells 7 or 13 days after induction of erythroblastic (left panels) or myelomonocytic (right panels) differentiation. Cells were analyzed by double-immunofluorescence flow cytometry after staining with a FITC-conjugated anti-LNGFR antibody and PE-conjugated antibodies against specific surface markers for erythroid (GpA, left panels) and myeloid (CD13, right panels) progeny. % values were indicate in each quadrant.

lane in Fig. 8.6 A). In fact, no viral β -globin signals were present in myeloid-differentiated CD34⁺ cells (fourth lane in Fig. 8.6 A) and, as expected, in mock-transduced cells (first and second lane in Fig. 8.6 A). This confirmed the erythroid specific expression of the GATA-LTR in transduced human HSCs. Quantitative experiments were performed to determine the proportion of viral β -globin expression with respect to the expression of endogenous globins. Viral β -globin transcripts accumulated in erythroid-differentiated cells at a level of 2.2% with respect to the endogenous α and β -globins, as quantified by phosphorimaging of Northern blots after normalization for GAPDH mRNA content (Fig. 8.6 A, bottom panel).

The activity of the G. β .W/P. Δ N vector was characterized at the protein level by Western blot analysis of the same bulk cultures of mock-transduced and transduced CD34⁺ cells, after induction of erythroid or myeloid differentiation (Fig. 8.6 B). As with the stable cell lines, a FLAG-modified human β -globin was used to distinguish viral β -globin signal from the endogenous globins. Protein analysis was performed using a polyclonal antibody against all the human globin chains. As expected, no endogenous globin signal was detected in myeloid-differentiated CD34⁺ cells (second lane in Fig. 8.6 B), while a signal, quantified at a level of 3.4% with respect to endogenous globins, was detected in transduced, erythroid-differentiated CD34⁺ cells (third lane in Fig. 8.6 B). Obviously, no viral human β -globin signal was detected in mock-transduced, erythroid-differentiated CD34⁺ cells (first lane in Fig. 8.6 B). As before, the specificity of the viral human β -globin signal was confirmed by Western blot analysis of erythroid-differentiated CD34⁺ cells using a monoclonal antibody specific for the FLAG epitope (8.6 B, lower panel).

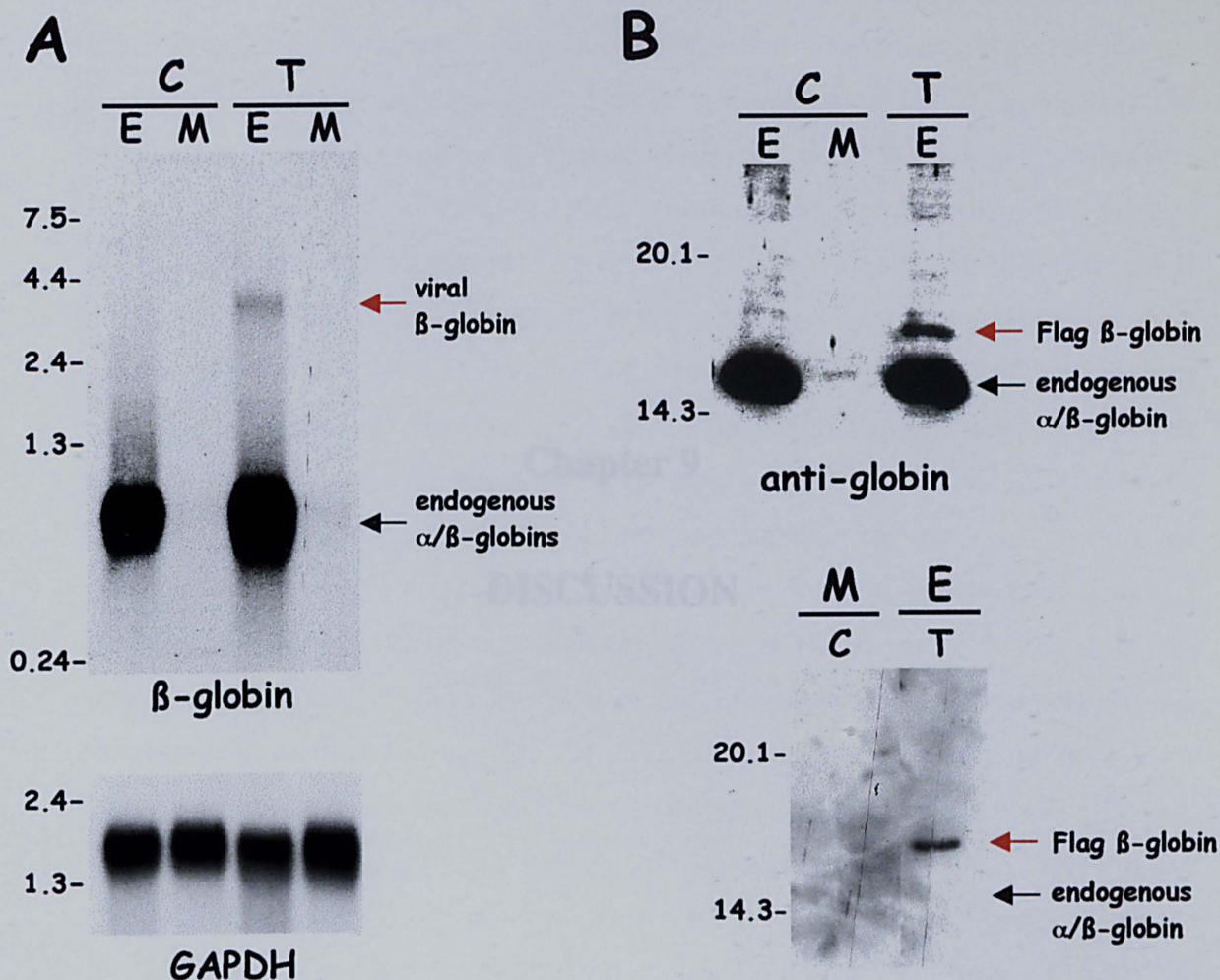


Figure 8.6. (A) Northern blot analysis of total RNA extracted from mock-transduced (C) and G. β .W/P. Δ N-transduced (T) CD34⁺ cells, 13 days after induction of erythroid (E) or myeloid (M) differentiation. The membrane was hybridised to a β -globin (upper panel) or a GAPDH (lower panel) probe. Viral and endogenous globin bands are indicated on the right. (B) Western blot analysis of proteins extracted from the same cells revealed using a polyclonal anti-human globin or a monoclonal anti-FALAG antibody. Bands relative to the Flag- β -globin and to the endogenous α/β globins are indicated on the right. Molecular weights in kilobase (A) and in kilodalton (B) are shown on the left.

Chapter 9

DISCUSSION

The prospect of effectively transducing HSCs creates a number of potential novel opportunities for the treatment of inherited and acquired diseases (Bordignon and Roncarolo, 2002). However, the successful implementation of stem cell gene therapy will require safe and efficient gene transfer procedures as well as controlled and regulated transgene expression. Lentiviral vectors are emerging as the gene delivery system of choice for transducing HSCs without compromising their repopulating capacity after BM transplantation (Follenzi et al., 2000; Miyoshi et al., 1999; Woods et al., 2000). Until now, the investigation of transgene regulation in hematopoietic cells has mostly focused on non-specific promoter/enhancer elements. These include elements of viral origin, such as retroviral LTRs and mammalian promoters. These promoters function in all hematopoietic lineages, albeit with variable strength. Accordingly, current clinical applications are limited to diseases where non-specific and relatively low-level expression of a vector-encoded product is sufficient. In disorders such as adenosine deaminase deficiency, Gaucher's disease or X-linked severe combined immunodeficiency (SCID), it seems acceptable to indiscriminately express the transgene product in lymphoid and myeloid lineages, irrespective of whether the pathology prevails in, for example, T lymphocytes or macrophages (Cavazzana-Calvo et al., 2000); Aiuti et al., 2002; Cavazzana-Calvo and Hacein-Bey-Abina, 2001). In the case of enzyme deficiencies, expression of a small fraction of physiological enzyme levels tends to suffice to yield therapeutic responses. In these settings, the transcriptional constraints for achieving clinical benefits without toxicity due to overexpression or ectopic expression are thus favourable. However, gene therapy of most blood genetic disorders (for example, chronic granulomatous disease and thalassemia) requires *ex vivo* gene transfer transplantable, self-renewing HSCs and regulation of transgene expression in one or more differentiated cell lineages (Sadelain, 2002). At present, there are very few reports on how to deliver the gene product specifically to only one lineage, differentiating from the transduced HSCs, or how to regulate the level of expression or its differentiation stage specificity within a single lineage. Targeted transduction could theoretically be achieved by manipulating the vector envelope, so that binding and infection is confined to a specific cell lineage. However, this form of targeting has proven ineffective so far (reviewed in Larochelle et al., 2002). To date, the only surface-targeting strategies that have allowed efficient infection by retroviral vectors *in vivo* are those in which the normal retroviral surface protein (SU) participates in receptor binding and fusion (Martin et al., 2002; Peng et al.,

1999). However, while the potential of this virus-entry targeting approach could have a clear impact on cancer gene therapy, it seems less attractive for clinical application requiring a lineage-restricted expression of the therapeutic gene. On the other hand, transcriptional targeting by insertion of regulatory sequences into the vector to control transcription and confine expression of the transgene to a specific lineage after stem cell differentiation looks more promising. The work of this PhD thesis attempted to address basic issues in stem-cell gene therapy from the perspective of regulating transgene expression, taking globin gene transfer for the treatment of β -thalassemia as a paradigm.

One way to target transgene transcription is to drive an internal expression cassette with specific enhancer/promoter combinations. With this simple design, expression of a reporter or a therapeutic transgene has been effectively restricted to erythroblasts, antigen presenting cells and, very recently, to CD4⁺ cells derived from murine or human HSCs (Cui et al., 2002; Marodon et al., 2003; Moreau-Gaudry et al., 2001; Richard et al., 2001). For some gene therapy applications, for example, the correction of globin chain imbalance in human β -thalassemia, a high protein output is just as important as its restricted expression, which makes practically inadequate the use of intronless β -globin cDNAs expressed by internal promoters. This problem has been partially resolved by placing the entire human β -globin gene under the control of its own promoter and a reduced version of the β -globin locus control region (LCR) in opposite orientation within a first-generation LV vector. With this design, potentially therapeutic levels of human β -globin have been obtained for the first time in a murine model of β -thalassemia (May et al., 2000; May et al., 2002; Rivella et al., 2003).

As an alternative strategy of transcriptional targeting, we have attempted to re-direct the activity of the HIV-1 promoter in our lentiviral vector by LTR enhancer replacement (Lotti et al., 2002). Most of the U3 region of the HIV-1 3'LTR (from position -418 to -40 from the transcription start site) has been deleted and replaced with a 200-bp auto-regulatory enhancer (HS2) of the erythroid-specific GATA-1 transcription factor (Tsai et al., 1989). In this design, the modified GATA-LTR controls the expression of a GFP cDNA as a reporter gene. Analysis of GFP expression showed the erythroid lineage specificity of the GATA-LTR upon transduction of a large panel of human and murine erythroid and non-erythroid cell lines (see Figs. 4.3 and 4.4).

As a general strategy, modification of the viral LTR offers a number of potential advantages that have been addressed one by one in this study. Firstly, the LTR enhancer replacement provides a more efficient vector design, which allows the use of the major viral transcription unit to express the gene of interest under the form of a genomic, spliceable RNA. This is a particularly suitable feature in gene therapy vectors aimed to clinical applications, such as human β -thalassemia, where a high protein output is as important as its restricted expression. In order to test the efficacy of this advantage, we have directly compared the expression of the GFP reporter gene driven by the modified GATA-LTR (in the G vector) with that obtained by the same enhancer/promoter combination placed in the conventional internal position in the context of a SIN lentiviral vector (in the -18/G vector). Transduction of HEL cells with the two different vectors showed that expression from the genomic transcript is higher than that obtained from an unspliced transcript driven by an internal promoter (see Figs. 5.1 A and 5.2).

Secondly, the presence of two copies of a genomic enhancer flanking the integrated provirus could reduce the chances of chromatin-mediated inactivation of transcription, which is known to affect the long-term maintenance of retroviral transgene expression *in vivo*, particularly in stem cells (Challita and Kohn, 1994). Expression of integrated provirus is affected by chromatin structure. Because the bulk of the mammalian genome is packaged into transcriptionally silent heterochromatin (Karpen, 1994), and murine leukemia virus-based vectors insert at random sites in the genome, a large portion of murine leukemia virus insertions result in gene silencing. This can lead to highly variable expression among clones, with complete silencing of provirus expression in a significant fraction of clones either immediately after insertion or after following cell expansion. The progeny of a single clone containing a unique integration event can also be affected by the surrounding chromatin to varying degrees (Karpen, 1994), a phenomenon known as position effect variegation (PEV). The mammalian genome is organised into discrete chromosomal domains, in part using sequences termed chromatin insulators (Bell and Felsenfeld, 1999). These elements, first described in *Drosophila* and more recently in several vertebrate species, help define the boundary between differentially regulated loci and serve to shield promoters from the influence of neighbouring regulatory elements (Prioleau et al., 1999). Insulators function in a polar manner (e.g., they must be located between the *cis* effectors and promoter) and do not

have stimulatory or inhibitory transcriptional effects on their own, distinguishing them from classical enhancers and silencers. The first and best-characterised vertebrate chromatin insulator is located within the chicken β -globin locus control region. This element, which contains a DNase-I hypersensitive site (cHS4), appears to constitute the 5' boundary of the chicken β -globin locus. A 1.2-kb fragment containing the cHS4 element displays classic insulator activities, including the ability to block the interaction of globin gene promoters and enhancers in cell lines (Chung et al., 1993), and the ability to protect expression cassettes in *Drosophila* and transgenic mammals from position effects. It is noteworthy though that tandem flanking copies of the 5'HS4 element are required for full insulating activity (Chung et al., 1993). Other elements exist in the eukaryotic genome that are believed to contribute toward delimiting the topological borders between chromatin domains (Bode et al., 1992). Termed scaffold or matrix attached regions (S/MARs), these elements have been proposed to anchor chromatin to a skeleton of protein cross-ties called the nuclear scaffold (in metaphase) or nuclear matrix (in interphase), forming chromosomal loops (Namciu et al., 1998; Schubeler et al., 1996). It has been proposed that S/MARs may function by bringing enhancers and others distal regulatory elements in close proximity to their corresponding promoter sequences. While insulator function has been attributed to some S/MARs, this does not appear to be a general property of these elements (Namciu et al., 1998). Based on the assumption that insulator elements may play a useful role in attenuating chromatin position effects, a series of retroviral and lentiviral vectors carrying these elements were generated (Agarwal et al., 1998; Auten et al., 1999; Dang et al., 2000; Emery et al., 2000; Rivella et al., 2000). While all the reports showed an improved performance in gene expression of the viral vectors, these elements fail in shielding transgene expression from transcriptional silencing (Emery et al., 2002; Murray et al., 2000; Rivella et al., 2000). Very recently, by combinatorial association of the 5' cHS4 insulator and the human interferon- β scaffold attachment region (INT-SAR), position-effect protection of LV vector-mediated transgene expression in human KG1a hematopoietic progenitor cell line and in primary human CD34+ has been reported (Ma et al., 2003; Ramezani et al., 2003). However, two findings seem to reshape the relevance of these data. Firstly, the introduction of GFP+ cell sorting by FACS during KG1a cells cloning would eliminate integration sites where viral expression is silenced or very weak, introducing a non-acceptable bias in this type of analysis (Rivella and Sadelain, 1998). Secondly, KG1a clonal analysis showed the persistence of a high

variability in transgene expression also in the presence within the vector of the 5'HS4 and INF-SAR. The predominant effect of these two elements is in the increase of the mean fluorescence intensity (MFI) of the transgene. Persistence of expression reported by Ramezani *et al.* could be explained by the overall enhancement in transgene expression of 5'HS4/INF-SAR-carrying clones (Ramezani *et al.*, 2003). Nevertheless, suppression of repressive position effects by transcriptional enhancer has been extensively reported (Francastel *et al.*, 1999; Sutherland *et al.*, 1997; Walters *et al.*, 1996). Chromatin opening by pioneer transcription factors may be an integral part of transcriptional activation in a situation, such as cell differentiation, where silencing is used as a means of gene regulation (Francastel *et al.*, 2000; Martin, 2001). This function in some ways resembles chromatin insulation. Indeed, very recently, chromatin insulation by a transcriptional activator has been reported for a GFP reporter construct carrying multiple binding sites for a metal-inducible activator MTF (Sutter *et al.*, 2003). Induction makes silent transgenes more likely to become active and active transgenes less likely to become silent. Thus, the transcriptional activator suppresses both stable and variegating position effects in a manner that resembles chromatin insulation (Sutter *et al.*, 2003). Binding of erythroid-specific transcription factors, such as GATA-1, to enhancers of erythroid-specific genes early in development or differentiation could be a key factor in initiation and maintenance of active chromatin structures (Martin *et al.*, 1996). GATA-1 orchestrates multiple programs of erythroid development, including control of the cell cycle and apoptosis, as well as differentiation (see Section 1.4.3.1). At the biochemical level, GATA-1 is thought to recruit critical proteins to target sites. The interaction of GATA-1 with CBP/p300 suggests at least one mechanism by which histone acetylases might be brought to specific site (Blobel, 2000; Blobel *et al.*, 1998). By modifying chromatin-bound histones or perhaps by modification of GATA-1 itself, acetylases could enhance transcriptional activity of erythroid-specific loci (Boyes *et al.*, 1998). In this context, GATA-1 might be viewed more as an “architectural” factor rather than a traditional transcriptional activator. Very recently, the essential role of GATA-1 and its cofactor CBP for the formation of an erythroid-specific acetylation pattern that is permissive for high levels of gene expression has been reported (Letting *et al.*, 2003). Using chromatin immunoprecipitation (ChIP) experiments, Letting *et al.* found that GATA-1 stimulates acetylation of both histones H3 and H4 at the LCR and the β -major globin gene promoter. Time course experiments further demonstrated a correlation between GATA-1 occupancy at GATA elements, recruitment of CBP,

histone acetylation, and globin gene expression. These results suggest that one essential function of GATA-1 is to establish an erythroid-specific pattern of histone hyperacetylation at the globin gene cluster and very likely other erythroid-expressed genes (Letting et al., 2003). The ability of GATA-1 to disrupt a subset of histone-DNA contacts on a mononucleosome (Boyes et al., 1998) may relate to the ability of GATA-4, a similar protein enriched in the liver, to bind and open chromatin (Cirillo et al., 2002). The highly conserved zinc finger motif of this class of factors may generally possess a chromatin disrupting function.

All these observations provide a strong rationale for using transcriptionally targeted vectors based on GATA binding sites as described in this study, to restrict expression of transgenes to the erythroid lineage and to shield its expression from repressive effects of flanking chromatin. Indeed, our results show persistent expression driven by the GATA-LTR is at uniformly high levels in individual clones of transduced HEL cells, showing remarkably less position effect variegation than a vector based on the same enhancer/promoter combination placed in an internal position (see Chapter 5). This vector design could therefore be applicable in all cases where long-term persistence of gene expression, tight gene regulation and high-level protein production are required in specific progeny of transduced HSCs, such as in gene therapy of thalassemia.

Thirdly, another advantage of our transcriptional targeting design derives from the possibility to use an internal transcription unit to control a second gene providing an additional function to the transduced cells, generating bi-functional lentiviral vectors. For clinical applications, gene transfer vectors must be able to transduce HSCs at high efficiency without affecting their long-term repopulating capacity. Furthermore, since drastic myeloablation is an undesirable procedure for thalassemic patients, the vectors should carry an *in vivo* positive selection function to favour engraftment and expansion of genetically modified HSCs. The ideal vector for gene therapy of β -thalassemia should therefore drive constitutive expression of a positive selection function in long-term repopulating HSCs and restricted expression of therapeutic levels of β -globin in their erythroblastic progeny. These requirements complicate vector design, because the inclusion of a second transcription unit increases the size of the vector, may alter its genomic stability or decrease vector titer and the juxtaposition of two transcription units raises the possibility for transcriptional interference. Moreover, the two adjacent transcription units must function independently: one erythroid-specific and the other

pan-specific, directing expression in stem cells themselves and eventually their progeny in all lineages. All these issues have been addressed in this study by insertion of a second transcriptional cassette, containing the Δ LNFR cDNA under the control of a constitutive PGK promoter, downstream of the GFP gene in both G and -40 vectors. In this design, the modified GATA-LTR controls expression of the gene of interest (GFP or β -globin in this study), while the internal PGK promoter drives the constitutive expression of a second reporter gene (Δ LNFR). Insertion of the second transcriptional cassette had no effect on titer, infectivity and genomic stability of the vector (see Fig. 6.2). Gene expression analysis carried out on cells transduced with the G/P vector showed that the two transgenes are independently expressed in an erythroid-specific fashion (GFP) or constitutively in all cell lineages (Δ LNFR). Unfortunately, GFP expression analysis of the same cells revealed that the internal promoter negatively interferes with the activity of the targeted LTR, thereby reducing the overall output of the vector genomic transcript with respect to single-gene vectors. The cause of this phenomenon comes from possible transcriptional interference between the two enhancer/promoter elements in the vector or from a reduced stability of the genomic transcripts. Both mechanisms can influence accumulation of the genomic transcripts in infected cells. Although transcriptional interference could partially account for this reduction on genomic transcript accumulation, mRNA stability could also play an important role to this outcome. This possibility arises from the observation that the genomic transcript contains an internal stop codon, which could mimic a premature translation termination codon. All eukaryotes possess the ability to detect and degrade transcripts harbouring premature signals for the termination of translation (Lykke-Andersen, 2001). This surveillance mechanism, known as nonsense-mediated decay (NMD), is thought to protect the organism from the deleterious effects of truncated proteins that could result if nonsense transcripts were stable. Thus, genomic transcripts could be prone to NMD and degraded soon after transcription. Nevertheless, human β -globin mRNA decay in erythroid cells has been reported (Stevens et al., 2002). Another step that plays a crucial role in transcript stability during mRNA processing is the efficiency of polyadenylation. Since in all retroviruses transcript polyadenylation occurs within the 3'LTR, the insertion of a transcriptional cassette between the 3'LTR and the reporter gene could reduce its efficiency.

Despite the mechanisms responsible of this phenomenon, we tried to overcome this problem by introducing a regulatory sequence from the genome of the woodchuck hepatitis virus (WPRE) at different positions within the vector construct (see Fig. 6.5A). This element acts at a post-transcriptional level, most likely promoting the efficiency of polyadenylation of the nascent transcript and increasing its total amount in cells (Donello et al., 1998). A WPRE positioned at the 3' end of the vector was transcribed in both the genomic and the sub-genomic RNA and accordingly increased expression of both GFP and Δ LNGFR. Conversely, a WPRE downstream of the GFP gene but upstream of the PGK promoter was incorporated only in the genomic transcript, and caused a selective increase of the GATA-LTR-driven GFP expression with no effect on its transcriptional restriction. Use of a WPRE therefore allows modulation of the relative abundance of two different gene products within a single lentiviral vector, and to restore high-level expression of a modified LTR even in the presence of an interfering internal promoter (6.5 B).

Human cord blood-derived CD34⁺ stem/progenitor cells and murine repopulating HSCs were transduced at high efficiency (up to 70%) with this type of vector. This is shown by the expression of the constitutively regulated reporter gene in undifferentiated progenitors as well as in differentiated progenies (myeloid, lymphoid and erythroid) obtained *in vitro* and *in vivo* upon transplantation into NOD/SCID (human cells) or co-isogenic (murine cells) mice. Expression of the transgene driven by the modified LTR was successfully restricted to the erythroblastic progeny of CD34⁺ progenitors *in vitro* and of human SCID-repopulating cells and murine HSCs *in vivo*. Transcription from the chimeric GATA-LTR was active at low levels in a subpopulation of transduced CD34⁺ cells (probably cycling progenitors), down regulated in myelo-monocytic cells and up regulated and maintained at sustained levels in erythroblastic cells, strictly paralleling the expression pattern of the GATA-1 gene during differentiation of human primary hematopoietic cells (Sposi et al., 1992). Nevertheless, the GATA-LTR expression potential is maintained throughout differentiation, from transduced hematopoietic cells to erythroid cell, even after 9 months *in vivo*. These results strongly suggest that the vector-encoded GFP expression resists transgene silencing at almost all integration sites (see Fig. 7.9). Interestingly, expression from the hybrid LTR does not require co-expression of TAT and therefore appears to be relatively insensitive to the presence of a TAR secondary structure in the genomic transcript produced by the integrated provirus.

Overall, these experiments show that in our vectors the internal promoter is transcribed at approximately constant levels in undifferentiated HSCs and all their differentiated progeny *in vitro* and *in vivo* and that transcription from the modified LTR is effectively restricted to the erythroblastic lineage. This design is therefore suitable for expressing a constitutive *in vivo* selection marker, instead of the Δ LNGFR gene, and a transcriptionally targeted therapeutic gene, instead of the GFP gene, in a single vector.

Transcription factors, regulators of cell survival or cell proliferation may be used to enhance HSC functions. These novel approaches may be helpful in controlling post-transplantation expansion and maybe in increasing donor chimerism in non-myelo-ablated recipients (Sadelain et al., 2000). However, the challenge is to enhance HSC function without impairing differentiation, without accelerating HSC exhaustion and without favouring leukemogenesis. Among the numerous *in vivo* selection strategies, a promising approach is the use of genes encoding modified growth factor receptors conferring ligand-dependent, selective growth advantage to transduced cells. A number of recent reports indicate that reasonable candidates for this function are the receptors for hematopoietic growth factors and particularly that of erythropoietin (EpoR). Deletion of the carboxyl-terminal 91 amino acids of EpoR enhances the sensitivity of the receptor for the ligand, leading to an increase in the proliferative response to Epo stimulation in cells expressing this variant (D'Andrea and Koo, 1991). Transgenic mice carrying the tEpoR gene are phenotypically normal, but show an increase in multipotent clonogenic progenitors upon administration of exogenous Epo (Kirby et al., 1996). Competitive repopulating experiments carried out by transplanting BM from tEpoR transgenic mice into wild-type recipients showed that stem cells expressing tEpoR have a selective growth advantage over wild-type cells in Epo-treated animals (Kirby et al., 2000). Interestingly, similar naturally occurring EpoR mutants have been reported in humans, where they cause benign erythroid proliferation but no malignant transformation (Arcasoy et al., 1997; de la Chapelle et al., 1993; Watowich et al., 1999). The tEpoR gene appears therefore to be non-toxic, non-tumorigenic and non immunogenic in both mice and men. Nevertheless, it remains to be established whether such beneficial properties are maintained when the transgene is introduced into the genome of HSCs in the context of a viral vector, and what type of regulation is required

to provide appropriate ligand response and selective advantage to transduced cells upon transplantation *in vivo*.

An inherent limitation of our vector design is the persistence of a potentially active, TAT-responsive HIV promoter in the integrated provirus. The TAT protein binds to the TAR region in the nascent genomic transcript and tethers the cyclin T-CDK9 complex to polymerase II, leading to increased transcriptional elongation (Wei et al., 1998). By this action, TAT acts as a transcriptional activator and therefore plays a pivotal role in the exceedingly high replication rates that characterise HIV-induced diseases (Cullen, 1998). The chimeric GATA-LTR HIV enhancer/promoter could theoretically be activated by super infection of wild-type HIV, for instance in the T cell or macrophage progeny of transduced HSCs, thereby leading to mobilisation of the vector genome into infective HIV particles. Although the occurrence of such an event should be practically verified in an appropriate *in vivo* model, vector mobilisation would be an undesirable effect, potentially affecting the safety characteristics of the gene transfer system for clinical applications. However, the latest generation of lentiviral packaging systems no longer rely on TAT for expression of the transfer vector into the packaging cell line (Klages et al., 2000). This allows a design of lentiviral vectors, which do not incorporate a TAR element in the primary transcript. Mutation of the TAR element downstream the targeted LTR should abolish the possibility of HIV-induced mobilisation of the vector and therefore restore a degree of safety comparable to that of the currently used, fully U3-deleted SIN vectors.

The β -thalassaemias are a group of inherited anaemias characterised by a reduction or a total absence in the production of haemoglobin β -chains (Weatherall, 2001; Weatherall, 2001). The resulting imbalance of β -globin to α -globin chains results in a severe anemia that is generally treated by repeated blood transfusions. A consequence of such regular blood transfusion is the increased risk of exposure to virus-infected blood supplies and eventually leads to iron overload that can cause organ failure unless treated by iron chelation drug therapy (Olivieri, 1999; Olivieri et al., 1998). The only existing cure is allogeneic bone marrow transplantation (BMT), which is however available for less than 30% of thalassemic patients (Giardini and Lucarelli, 1994). Gene therapy is an attractive potential alternative to BMT (Sorrentino and Nienhuis, Gene Therapy for hematopoietic diseases. In: Stamatoyannopoulos G *et al.*, 2001).

Gene therapy of thalassaemias requires a restricted expression of a globin transgene only to the erythropoietic progeny of the hematopoietic stem cell and represents one of the most challenging models to test the efficiency and efficacy of transcriptional targeting of viral vectors. The clinical history of the disease and over 15 years of BMT experience indicate that even a mild correction ($>15\%$) of the globin chain imbalance in a fraction ($>25\%$) of maturing erythroblasts is sufficient to reduce the morbidity caused by ineffective erythropoiesis, to improve the clinical management of the disease and to increase the patients' life expectancy (Gaziev and Lucarelli, 2003). For therapeutic purposes, reproducing the full pattern of the complex regulation of β -globin gene expression might be unnecessary. However, the ideal β -globin gene therapy vector should stably integrate into an HSC at high efficiency and be expressed to near endogenous levels at single copy. Position-dependent silencing and position effect variegation are particularly troublesome for retrovirus vectors containing the human β - or γ -globin genes (Raftopoulos et al., 1997; Rivella and Sadelain, 1998). Over the years, a number of scientists have tried to obtain lineage-restricted, high-level transcription of the β -globin gene under control of minimal sized (200-300bp) LCR elements within the framework of MoMLV-derived retroviral (Sadelain et al., 1995) or adeno-associated viral vectors (Einrhand *et al.*, 1995) (Dong et al., 2002; Tang and Qian, 1998). Expression studies in retrovirally transduced cells demonstrated that β -globin vectors harbouring a 1.0-kb LCR element, made up of the juxtaposed HS2, HS3, and HS4 core elements, could enhance the average β -globin expression in an erythroid-specific fashion, but failed to reduce positional variability of expression (Sadelain et al., 1995).

Moreover, *in vivo* studies in long-term bone marrow chimeras transplanted with β -globin vectors harbouring the HS2, HS3, and HS4 core sites, demonstrated that this minimal LCR failed to sustain elevated and long-term expression (Raftopoulos et al., 1997; Rivella and Sadelain, 1998). Thus, the data suggested that the 1.0-kb LCR merely functioned like a classic enhancer rather than a chromatin regulator able to confer position-independent expression. The *in vivo* data showed relatively low (less than 3% of an endogenous globin gene), variable, and rarely persistent levels of vector-encoded human β -globin expression. Likewise, the results obtained with AAV vectors harbouring globin genes linked to LCR elements have been disappointing. Furthermore, the ability of AAV to integrate efficiently into hematopoietic stem cells has been controversial, with conflicting results reported from a number of groups (Russell and Kay, 1999). The results achieved with the minimal 1.0-kb LCR are consistent with transgenic studies that show the hypersensitive site cores are necessary for expression, but require additional sequences lying outside the hypersensitive site cores for full LCR function (Hardison et al., 1997). In an effort to improve upon previous vectors, attempts have been made to effectively increase the size of the LCR element, incorporating the LCR core elements along with additional flanking sequences. Unfortunately, incorporation of the larger genomic fragments in retroviral vectors proved problematic. This was due in part to the inability of vectors to incorporate large LCR elements in a stable manner. Vector rearrangements frequently ensued, precluding introduction of intact unrearranged copies of the β -globin transcription unit into target cells at a high frequency. The difficulty to transfer large genomic sequences by using oncoretroviral vector systems, such as MLV-derived vectors, has limited their usefulness in gene therapy approaches to treat severe hemoglobin disorders. The combined effect of high gene transfer efficiency, and absence of vector rearrangements afforded by the recombinant lentivirus harboring the β -globin gene and LCR configuration, yielded remarkably high levels of human β -globin expression. In fact, the synthesis of potentially therapeutic levels of human β -globin was achieved in the erythrocytes of bone marrow chimeras transplanted with β -thalassemic bone marrow or foetal liver cells, transduced with the 1st generation lentiviral vector TNS9 (May et al., 2000; May et al., 2002). The TNS9 lentiviral vector contains the human β -globin gene, including extended promoter sequences, the β -globin 3' proximal enhancer and LCR elements HS2, 3 and 4 as a 3.2kb combination, cloned in opposite orientation in respect to HIV LTR transcriptional direction. Although these results showed that appropriate regulation

of a globin gene can be technically achieved, intrinsic limitations to this system are the lacking of position-independent expression of virally encoded β -globin, demonstrated by clonal analysis of transduced MEL cells (May et al., 2000) and the size of the vector, which is unlikely to allow the insertion of a second transgene for selection of transduced stem cells. Moreover, the TNS9 vector has been done in the backbone of a first-generation lentiviral vector containing two intact HIV-1 LTRs. A SIN version of the TNS9 lentiviral vector must be developed using the third-generation packaging system to prevent recombination events generating replication-competent HIV-1 virus.

Another alternative approach addressing the problem of vector silencing involves the use of selection schemes to enrich for stem or progenitor cells that have provirus integrated at transcriptionally permissive sites. In one application, Kalberer *et al* transduced mouse marrow with an oncoretrovirus vector for human β -globin and a GFP reporter gene and preselected GFP-expressing cells before transplantation by flow cytometry (Kalberer et al., 2000). As a result of preselection, they observed that the fraction of RBCs expressing GFP remained constant for up to 9.5 months after transplantation, and all mice expressed human β -globin in at least some peripheral RBCs. However, the levels of human β -globin expression were highly variable, and the published analysis did not include a serial assessment of human β -globin expression over time or a correlation between the fraction of RBCs expressing human β -globin and the level of provirus for individual animals. Thus, the effects of preselection on silencing of the therapeutic β -globin cassette in their study are difficult to assess.

A third approach to overcoming silencing of globin gene vectors involves replacing the promoter for the globin gene cassette with a enhancer/promoter from other genes known to be expressed at high levels in RBCs. In one promising application of this approach, Sabatino *et al* demonstrate that fusion of a minimal ankyrin promoter to a β -globin gene allowed for expression in a copy number-dependent fashion in transgenic mice and at low levels in virtually all peripheral RBCs following retrovirus vector-mediated transduction of bone marrow in mice, indicating that this cassette is relatively resistant to silencing and position effects (Sabatino et al., 2000; Sabatino et al., 2000). Adapting this last strategy to our transcriptional targeting design allowed us to exploit and benefit of the chromatin opening function attributed to pioneer transcription factor, such as GATA-1. Since our GATA-LTR vector possess all the safety features common to the latest generation of lentiviral vectors and seems to have a high probability to direct expression at all integration sites, using our targeting design we will probably be able to

overcome the major concerns regarding the ideal β -globin gene therapy vector. Based on this optimistic assumption we decided to develop GATA-LTR lentiviral for human β -globin expression.

The goal of our lentiviral vectors for β -globin expression was to reach a potentially therapeutic level of β -globin polypeptides (at least 10-20 % of that present in normal red blood cells) in thalassemic erythroblasts. On the basis of results obtained by using transcriptionally targeted lentiviral vectors expressing GFP under the control of GATA-LTR, we replaced the marker gene with a FLAG epitope tagged-440-bp β -globin cDNA. HIV-1 cPPT and WPRE sequences were added within the framework of the vector to increase the efficiency of target cell transduction and RNA stability. The FLAG epitope was cloned in frame at the 5' end of the β -globin cDNA to allow transgene detection with a specific anti-FLAG antibody. Gene expression obtainable with these modifications was measured at the level of RNA accumulation by Northern blot analysis and protein synthesis by Western blot analysis.

HEL and MEL cells, expressing endogenous human α/γ and murine α/β globins respectively, were transduced with lentiviral vectors and induce to differentiate in the presence of 50 μ M hemin and 2% DMSO respectively. The results from the Northern blot analysis showed that in hemin-induced HEL cells, the viral β -globin transcripts accumulated at 230% of the level of endogenous globin mRNAs. The same analysis in DMSO-induced MEL cells showed that the viral β -globin signal was 70% with respect to the endogenous murine globin signal. Similar results were obtained with the TNS9 lentiviral vector by May and colleagues who reported that, after normalization to vector copy number and to endogenous β -globin expression per allele, human β -globin levels were 71.3 ± 2.3 in pooled MEL cells (May et al., 2000). Western blot analysis of hemin-induced HEL cells showed that Flag- β -globin was produced at a level of 42% with respect to endogenous human globin chains. In DMSO-induced MEL cells, the level of vector derived Flag- β -globin was 30% with respect to the endogenous murine signal.

The comparison of viral transcripts accumulated in induced and non-induced cells showed that the GATA-LTR is already activated in non-induced cells and down-regulated in induced cells. This suggests that the GATA-1 transcription factor is not involved in the late phase of erythroid differentiation in HEL and MEL cells. Therefore,

both HEL and MEL cells seem to be useful to study the activity of our vectors in systems with an inducible production of hemoglobin. However, within these cell lines our GATA-LTR vector does not appear to mimic the transcriptional activation dependence of GATA-1 observed during erythroid differentiation of the human HSCs. For this reason, we decided to test the activity of our G. β .W/p. Δ N vector upon transduction and differentiation of human HSCs *in vitro*. Results from Northern blot analysis after normalization to endogenous α and β -globin expression per allele, showed that viral β -globin transcripts in erythroid-differentiated CD34⁺ cells accumulated at a level of 8.8% with respect to the endogenous globins. At the protein level, a signal quantified as 3.4% of endogenous globins was detected in erythroid-differentiated CD34⁺ cells. After correction to endogenous α and β -globin expression per allele, the proportion of Flag- β -globin expression is 13.6%. Based on the data published from Persons and colleagues (Persons et al., 2001), a significant therapeutic benefit could be achieved with expression of a transferred gene at about 15% of the level of total α -globin in patients with severe β -thalassaemia in whom 20% of erythroid precursors express the vector genome. With our vector we are very near to this therapeutic level and these results are particularly encouraging because they were obtained using CD34⁺ cells from healthy donors, where a strong competition between viral and endogenous β -globin genes is present. For this reason, the real therapeutic efficacy of our vector should be tested in a β -thalassemic context. On the other hand, the full potential of our design remains unexplored, since the use of a second gene as an *in vivo* selectable marker could be crucial for the treatment of severe β -thalassaemia. Indeed, application of gene therapy approaches to treat severe β - is likely to occur initially within the context of minimal myeloablation. Treated individuals are therefore likely to have a mixture of a small minority of genetically modified autologous cells, in which vector-mediated globin transgene expression is significantly less than the output of the normal human β -globin gene locus, in a background with a majority of unmodified, diseased cells. With this approach, to provide an *in vivo* selection function to transduced cells could be essential.

In conclusion, we report a new lentiviral vector design based on the use of an enhancer-replaced, transcriptionally targeted HIV LTR to express a therapeutic gene and of a second, independently regulated internal promoter to drive the expression of an accessory function such as an *in vivo* selection marker. This vector combines the advantages of using the spliced, genomic viral transcript to obtain high-level protein expression with the possibility to direct gene expression in a tissue- or cell-specific function through the use of a cellular enhancer to restrict the activity of the LTR promoter. The possibility to insert a second, independently regulated, transcription cassette in this backbone renders such a vector design particularly suited for gene therapy applications based on genetic modification of pluripotent HSCs with a transcriptionally targeted transgene, in the context of a minimal or absent myeloablation of the patient.

Chapter 10

BIBLIOGRAPHY

Abruzzese, E., Radford, J. E., Miller, J. S., Vredenburg, J. J., Rao, P. N., Pettenati, M. J., Cruz, J. M., Perry, J. J., Amadori, S., and Hurd, D. D. (1999). Detection of abnormal pretransplant clones in progenitor cells of patients who developed myelodysplasia after autologous transplantation. *Blood* 94, 1814-9.

Agarwal, M., Austin, T. W., Morel, F., Chen, J., Bohnlein, E., and Plavec, I. (1998). Scaffold attachment region-mediated enhancement of retroviral vector expression in primary T cells. *J Virol* 72, 3720-8.

Aguila, H. L., Akashi, K., Domen, J., Gandy, K. L., Lagasse, E., Mebius, R. E., Morrison, S. J., Shizuru, J., Strober, S., Uchida, N., Wright, D. E., and Weissman, I. L. (1997). From stem cells to lymphocytes: biology and transplantation. *Immunol Rev* 157, 13-40.

Aiken, C. (1997). Pseudotyping human immunodeficiency virus type 1 (HIV-1) by the glycoprotein of vesicular stomatitis virus targets HIV-1 entry to an endocytic pathway and suppresses both the requirement for Nef and the sensitivity to cyclosporin A. *J Virol* 71, 5871-7.

Aiken, C., and Trono, D. (1995). Nef stimulates human immunodeficiency virus type 1 proviral DNA synthesis. *J Virol* 69, 5048-56.

Aiuti, A., Slavin, S., Aker, M., Ficara, F., Deola, S., Mortellaro, A., Morecki, S., Andolfi, G., Tabucchi, A., Carlucci, F., Marinello, E., Cattaneo, F., Vai, S., Servida, P., Miniero, R., Roncarolo, M. G., and Bordignon, C. (2002). Correction of ADA-SCID by stem cell gene therapy combined with nonmyeloablative conditioning. *Science* 296, 2410-3.

Akashi, K., Kondo, M., Cheshier, S., Shizuru, J., Gandy, K., Domen, J., Mebius, R., Traver, D., and Weissman, I. L. (1999). Lymphoid development from stem cells and the common lymphocyte progenitors. *Cold Spring Harb Symp Quant Biol* 64, 1-12.

Alexander, W. S. (1998). Cytokines in hematopoiesis. *Int Rev Immunol* 16, 651-82.

Andrews, N. C., Erdjument-Bromage, H., Davidson, M. B., Tempst, P., and Orkin, S. H. (1993). Erythroid transcription factor NF-E2 is a haematopoietic-specific basic-leucine zipper protein. *Nature* 362, 722-8.

Antonchuk, J., Sauvageau, G., and Humphries, R. K. (2002). HOXB4-induced expansion of adult hematopoietic stem cells ex vivo. *Cell* 109, 39-45.

Antoniou, M., de Boer, E., Spanopoulou, E., Imam, A., and Grosveld, F. (1995). TBP binding and the rate of transcription initiation from the human beta-globin gene. *Nucleic Acids Res* 23, 3473-80.

Antoniou, M., deBoer, E., Habets, G., and Grosveld, F. (1988). The human beta-globin gene contains multiple regulatory regions: identification of one promoter and two downstream enhancers. *Embo J* 7, 377-84.

- Antoniou, M., Geraghty, F., Hurst, J., and Grosveld, F. (1998). Efficient 3'-end formation of human beta-globin mRNA in vivo requires sequences within the last intron but occurs independently of the splicing reaction. *Nucleic Acids Res* 26, 721-9.
- Antoniou, M., and Grosveld, F. (1990). beta-globin dominant control region interacts differently with distal and proximal promoter elements. *Genes Dev* 4, 1007-13.
- Arcasoy, M. O., Degar, B. A., Harris, K. W., and Forget, B. G. (1997). Familial erythrocytosis associated with a short deletion in the erythropoietin receptor gene. *Blood* 89, 4628-35.
- Armstrong, J. A., Bieker, J. J., and Emerson, B. M. (1998). A SWI/SNF-related chromatin remodeling complex, E-RC1, is required for tissue-specific transcriptional regulation by EKLF in vitro. *Cell* 95, 93-104.
- Armstrong, J. A., and Emerson, B. M. (1996). NF-E2 disrupts chromatin structure at human beta-globin locus control region hypersensitive site 2 in vitro. *Mol Cell Biol* 16, 5634-44.
- Athanasiou, M., Mavrothalassitis, G., Sun-Hoffman, L., and Blair, D. G. (2000). FLI-1 is a suppressor of erythroid differentiation in human hematopoietic cells. *Leukemia* 14, 439-45.
- Auten, J., Agarwal, M., Chen, J., Sutton, R., and Plavec, I. (1999). Effect of scaffold attachment region on transgene expression in retrovirus vector-transduced primary T cells and macrophages. *Hum Gene Ther* 10, 1389-99.
- Balotta, C., Lusso, P., Crowley, R., Gallo, R. C., and Franchini, G. (1993). Antisense phosphorothioate oligodeoxynucleotides targeted to the vpr gene inhibit human immunodeficiency virus type 1 replication in primary human macrophages. *J Virol* 67, 4409-14.
- Baum, C. M., Weissman, I. L., Tsukamoto, A. S., Buckle, A. M., and Peault, B. (1992). Isolation of a candidate human hematopoietic stem-cell population. *Proc Natl Acad Sci U S A* 89, 2804-8.
- Bean, T. L., and Ney, P. A. (1997). Multiple regions of p45 NF-E2 are required for beta-globin gene expression in erythroid cells. *Nucleic Acids Res* 25, 2509-15.
- Behringer, R. R., Hammer, R. E., Brinster, R. L., Palmiter, R. D., and Townes, T. M. (1987). Two 3' sequences direct adult erythroid-specific expression of human beta-globin genes in transgenic mice. *Proc Natl Acad Sci U S A* 84, 7056-60.
- Bell, A. C., and Felsenfeld, G. (1999). Stopped at the border: boundaries and insulators. *Curr Opin Genet Dev* 9, 191-8.
- Bell, J. E. (1998). The neuropathology of adult HIV infection. *Rev Neurol (Paris)* 154, 816-29.
- Bickel, S., and Pirrotta, V. (1990). Self-association of the Drosophila zeste protein is responsible for transvection effects. *Embo J* 9, 2959-67.

Blobel, G. A. (2000). CREB-binding protein and p300: molecular integrators of hematopoietic transcription. *Blood* 95, 745-55.

Blobel, G. A., Nakajima, T., Eckner, R., Montminy, M., and Orkin, S. H. (1998). CREB-binding protein cooperates with transcription factor GATA-1 and is required for erythroid differentiation. *Proc Natl Acad Sci U S A* 95, 2061-6.

Bode, J., Kohwi, Y., Dickinson, L., Joh, T., Klehr, D., Mielke, C., and Kohwi-Shigematsu, T. (1992). Biological significance of unwinding capability of nuclear matrix-associating DNAs. *Science* 255, 195-7.

Bordignon, C., Notarangelo, L. D., Nobili, N., Ferrari, G., Casorati, G., Panina, P., Mazzolari, E., Maggioni, D., Rossi, C., Servida, P., and et al. (1995). Gene therapy in peripheral blood lymphocytes and bone marrow for ADA- immunodeficient patients. *Science* 270, 470-5.

Bordignon, C., and Roncarolo, M. G. (2002). Therapeutic applications for hematopoietic stem cell gene transfer. *Nat Immunol* 3, 318-21.

Boyes, J., Byfield, P., Nakatani, Y., and Ogryzko, V. (1998). Regulation of activity of the transcription factor GATA-1 by acetylation. *Nature* 396, 594-8.

Boyes, J., Omichinski, J., Clark, D., Pikaart, M., and Felsenfeld, G. (1998). Perturbation of nucleosome structure by the erythroid transcription factor GATA-1. *J Mol Biol* 279, 529-44.

Bukrinsky, M. I., and Haffar, O. K. (1997). HIV-1 nuclear import: in search of a leader. *Front Biosci* 2, d578-87.

Bulger, M., and Groudine, M. (1999). Looping versus linking: toward a model for long-distance gene activation. *Genes Dev* 13, 2465-77.

Bulger, M., Sawado, T., Schubeler, D., and Groudine, M. (2002). ChIPs of the beta-globin locus: unraveling gene regulation within an active domain. *Curr Opin Genet Dev* 12, 170-7.

Bungert, J., Dave, U., Lim, K. C., Lieuw, K. H., Shavit, J. A., Liu, Q., and Engel, J. D. (1995). Synergistic regulation of human beta-globin gene switching by locus control region elements HS3 and HS4. *Genes Dev* 9, 3083-96.

Burns, J. C., Friedmann, T., Driever, W., Burrascano, M., and Yee, J. K. (1993). Vesicular stomatitis virus G glycoprotein pseudotyped retroviral vectors: concentration to very high titer and efficient gene transfer into mammalian and nonmammalian cells. *Proc Natl Acad Sci U S A* 90, 8033-7.

Cantor, A. B., Katz, S. G., and Orkin, S. H. (2002). Distinct domains of the GATA-1 cofactor FOG-1 differentially influence erythroid versus megakaryocytic maturation. *Mol Cell Biol* 22, 4268-79.

- Cantor, A. B., and Orkin, S. H. (2001). Hematopoietic development: a balancing act. *Curr Opin Genet Dev* 11, 513-9.
- Carter, D., Chakalova, L., Osborne, C. S., Dai, Y. F., and Fraser, P. (2002). Long-range chromatin regulatory interactions in vivo. *Nat Genet* 32, 623-6.
- Cashman, J. D., and Eaves, C. J. (1999). Human growth factor-enhanced regeneration of transplantable human hematopoietic stem cells in nonobese diabetic/severe combined immunodeficient mice. *Blood* 93, 481-7.
- Caterina, J. J., Ciavatta, D. J., Donze, D., Behringer, R. R., and Townes, T. M. (1994). Multiple elements in human beta-globin locus control region 5' HS 2 are involved in enhancer activity and position-independent, transgene expression. *Nucleic Acids Res* 22, 1006-11.
- Caterina, J. J., Donze, D., Sun, C. W., Ciavatta, D. J., and Townes, T. M. (1994). Cloning and functional characterization of LCR-F1: a bZIP transcription factor that activates erythroid-specific, human globin gene expression. *Nucleic Acids Res* 22, 2383-91.
- Cavazzana-Calvo, M., Hacein-Bey, S., de Saint Basile, G., Gross, F., Yvon, E., Nussbaum, P., Selz, F., Hue, C., Certain, S., Casanova, J. L., Bousso, P., Deist, F. L., and Fischer, A. (2000). Gene therapy of human severe combined immunodeficiency (SCID)-X1 disease. *Science* 288, 669-72.
- Cavazzana-Calvo, M., and Hacein-Bey-Abina, S. (2001). Correction of genetic blood defects by gene transfer. *Curr Opin Hematol* 8, 360-7.
- Challita, P. M., and Kohn, D. B. (1994). Lack of expression from a retroviral vector after transduction of murine hematopoietic stem cells is associated with methylation in vivo. *Proc Natl Acad Sci U S A* 91, 2567-71.
- Charneau, P., Alizon, M., and Clavel, F. (1992). A second origin of DNA plus-strand synthesis is required for optimal human immunodeficiency virus replication. *J Virol* 66, 2814-20.
- Charneau, P., Mirambeau, G., Roux, P., Paulous, S., Buc, H., and Clavel, F. (1994). HIV-1 reverse transcription. A termination step at the center of the genome. *J Mol Biol* 241, 651-62.
- Chen, B. P., Galy, A. M., Fraser, C., and Hill, B. (1997). Delineation of the human hematolymphoid system: potential applications of defined cell populations in cellular therapy. *Immunol Rev* 157, 41-51.
- Chen, H., Ray-Gallet, D., Zhang, P., Hetherington, C. J., Gonzalez, D. A., Zhang, D. E., Moreau-Gachelin, F., and Tenen, D. G. (1995). PU.1 (Spi-1) autoregulates its expression in myeloid cells. *Oncogene* 11, 1549-60.
- Cheng, T., Rodrigues, N., Dombkowski, D., Stier, S., and Scadden, D. T. (2000). Stem cell repopulation efficiency but not pool size is governed by p27(kip1). *Nat Med* 6, 1235-40.

- Choi, O. R., and Engel, J. D. (1988). Developmental regulation of beta-globin gene switching. *Cell* 55, 17-26.
- Chung, J. H., Whiteley, M., and Felsenfeld, G. (1993). A 5' element of the chicken beta-globin domain serves as an insulator in human erythroid cells and protects against position effect in *Drosophila*. *Cell* 74, 505-14.
- Cirillo, L. A., Lin, F. R., Cuesta, I., Friedman, D., Jarnik, M., and Zaret, K. S. (2002). Opening of compacted chromatin by early developmental transcription factors HNF3 (FoxA) and GATA-4. *Mol Cell* 9, 279-89.
- Civin, C. I., Strauss, L. C., Brovall, C., Fackler, M. J., Schwartz, J. F., and Shaper, J. H. (1984). Antigenic analysis of hematopoiesis. III. A hematopoietic progenitor cell surface antigen defined by a monoclonal antibody raised against KG-1a cells. *J Immunol* 133, 157-65.
- Clackson, T. (2000). Regulated gene expression systems. *Gene Ther* 7, 120-5.
- Clever, J., Sasseti, C., and Parslow, T. G. (1995). RNA secondary structure and binding sites for gag gene products in the 5' packaging signal of human immunodeficiency virus type 1. *J Virol* 69, 2101-9.
- Coffin, J. M., Hughes, S. H., and Varmus, H. E. (1997). *Retroviruses*: Cold Spring Harbor Laboratory Press).
- Coghill, E., Eccleston, S., Fox, V., Cerruti, L., Brown, C., Cunningham, J., Jane, S., and Perkins, A. (2001). Erythroid Kruppel-like factor (EKLF) coordinates erythroid cell proliferation and hemoglobinization in cell lines derived from EKLF null mice. *Blood* 97, 1861-8.
- Collins, K. L., Chen, B. K., Kalams, S. A., Walker, B. D., and Baltimore, D. (1998). HIV-1 Nef protein protects infected primary cells against killing by cytotoxic T lymphocytes. *Nature* 391, 397-401.
- Crispino, J. D., Lodish, M. B., MacKay, J. P., and Orkin, S. H. (1999). Use of altered specificity mutants to probe a specific protein-protein interaction in differentiation: the GATA-1:FOG complex. *Mol Cell* 3, 219-28.
- Crispino, J. D., Lodish, M. B., Thurberg, B. L., Litovsky, S. H., Collins, T., Molkenin, J. D., and Orkin, S. H. (2001). Proper coronary vascular development and heart morphogenesis depend on interaction of GATA-4 with FOG cofactors. *Genes Dev* 15, 839-44.
- Cui, Y., Golob, J., Kelleher, E., Ye, Z., Pardoll, D., and Cheng, L. (2002). Targeting transgene expression to antigen-presenting cells derived from lentivirus-transduced engrafting human hematopoietic stem/progenitor cells. *Blood* 99, 399-408.
- Cullen, B. R. (1998). HIV-1 auxiliary proteins: making connections in a dying cell. *Cell* 93, 685-92.

- Custodio, N., Carmo-Fonseca, M., Geraghty, F., Pereira, H. S., Grosveld, F., and Antoniou, M. (1999). Inefficient processing impairs release of RNA from the site of transcription. *Embo J* 18, 2855-66.
- D'Andrea, A. D., and Koo, J. W. (1991). Structure and function of the erythropoietin receptor in stable erythropoietin-dependent transfectants. *Contrib Nephrol* 88, 48-56; discussion 57-8.
- Dang, Q., Auten, J., and Plavec, I. (2000). Human beta interferon scaffold attachment region inhibits de novo methylation and confers long-term, copy number-dependent expression to a retroviral vector. *J Virol* 74, 2671-8.
- de la Chapelle, A., Traskelin, A. L., and Juvonen, E. (1993). Truncated erythropoietin receptor causes dominantly inherited benign human erythrocytosis. *Proc Natl Acad Sci U S A* 90, 4495-9.
- deBoer, E., Antoniou, M., Mignotte, V., Wall, L., and Grosveld, F. (1988). The human beta-globin promoter; nuclear protein factors and erythroid specific induction of transcription. *Embo J* 7, 4203-12.
- Delvoye, N. L., Destroismaisons, N. M., and Wall, L. A. (1993). Activation of the beta-globin promoter by the locus control region correlates with binding of a novel factor to the CAAT box in murine erythroleukemia cells but not in K562 cells. *Mol Cell Biol* 13, 6969-83.
- Dexter, T. M., and Heyworth, C. M. (1994). Growth factors and the molecular control of haematopoiesis. *Eur J Clin Microbiol Infect Dis* 13, S3-8.
- Domen, J., and Weissman, I. L. (2000). Hematopoietic stem cells need two signals to prevent apoptosis; BCL-2 can provide one of these, Kitl/c-Kit signaling the other. *J Exp Med* 192, 1707-18.
- Donahue, R. E., Kessler, S. W., Bodine, D., McDonagh, K., Dunbar, C., Goodman, S., Agricola, B., Byrne, E., Raffeld, M., Moen, R., and et al. (1992). Helper virus induced T cell lymphoma in nonhuman primates after retroviral mediated gene transfer. *J Exp Med* 176, 1125-35.
- Donahue, R. E., Kirby, M. R., Metzger, M. E., Agricola, B. A., Sellers, S. E., and Cullis, H. M. (1996). Peripheral blood CD34+ cells differ from bone marrow CD34+ cells in Thy-1 expression and cell cycle status in nonhuman primates mobilized or not mobilized with granulocyte colony-stimulating factor and/or stem cell factor. *Blood* 87, 1644-53.
- Donello, J. E., Beeche, A. A., Smith, G. J., 3rd, Lucero, G. R., and Hope, T. J. (1996). The hepatitis B virus posttranscriptional regulatory element is composed of two subelements. *J Virol* 70, 4345-51.
- Donello, J. E., Loeb, J. E., and Hope, T. J. (1998). Woodchuck hepatitis virus contains a tripartite posttranscriptional regulatory element. *J Virol* 72, 5085-92.

- Dong, W. J., Wu, X. B., Liu, D. P., Li, J. L., Liu, G., Zu, Z. X., Zhao, N., Hou, Y. D., and Liang, C. C. (2002). Analysis of adeno-associated virus-mediated ex vivo transferred human beta-globin gene in bone marrow engrafted mice. *J Biomed Sci* 9, 253-60.
- Donze, D., Townes, T. M., and Bieker, J. J. (1995). Role of erythroid Kruppel-like factor in human gamma- to beta-globin gene switching. *J Biol Chem* 270, 1955-9.
- Dull, T., Zufferey, R., Kelly, M., Mandel, R. J., Nguyen, M., Trono, D., and Naldini, L. (1998). A third-generation lentivirus vector with a conditional packaging system. *J Virol* 72, 8463-71.
- Dunaway, M., and Droge, P. (1989). Transactivation of the *Xenopus* rRNA gene promoter by its enhancer. *Nature* 341, 657-9.
- Dunbar, C. E., Seidel, N. E., Doren, S., Sellers, S., Cline, A. P., Metzger, M. E., Agricola, B. A., Donahue, R. E., and Bodine, D. M. (1996). Improved retroviral gene transfer into murine and Rhesus peripheral blood or bone marrow repopulating cells primed in vivo with stem cell factor and granulocyte colony-stimulating factor. *Proc Natl Acad Sci U S A* 93, 11871-6.
- Dzierzak, E., and Medvinsky, A. (1995). Mouse embryonic hematopoiesis. *Trends Genet* 11, 359-66.
- Dzierzak, E., Medvinsky, A., and de Bruijn, M. (1998). Qualitative and quantitative aspects of haematopoietic cell development in the mammalian embryo. *Immunol Today* 19, 228-36.
- Ellis, J., Talbot, D., Dillon, N., and Grosveld, F. (1993). Synthetic human beta-globin 5'HS2 constructs function as locus control regions only in multicopy transgene concatamers. *Embo J* 12, 127-34.
- Ellis, J., Tan-Un, K. C., Harper, A., Michalovich, D., Yannoutsos, N., Philipsen, S., and Grosveld, F. (1996). A dominant chromatin-opening activity in 5' hypersensitive site 3 of the human beta-globin locus control region. *Embo J* 15, 562-8. [abs.html](#).
- Ema, H., Takano, H., Sudo, K., and Nakauchi, H. (2000). In vitro self-renewal division of hematopoietic stem cells. *J Exp Med* 192, 1281-8.
- Emery, D. W., Yannaki, E., Tubb, J., Nishino, T., Li, Q., and Stamatoyannopoulos, G. (2002). Development of virus vectors for gene therapy of beta chain hemoglobinopathies: flanking with a chromatin insulator reduces gamma-globin gene silencing in vivo. *Blood* 100, 2012-9.
- Emery, D. W., Yannaki, E., Tubb, J., and Stamatoyannopoulos, G. (2000). A chromatin insulator protects retrovirus vectors from chromosomal position effects. *Proc Natl Acad Sci U S A* 97, 9150-5.
- Enver, T., and Greaves, M. (1998). Loops, lineage, and leukemia. *Cell* 94, 9-12.

- Enver, T., Raich, N., Ebens, A. J., Papayannopoulou, T., Costantini, F., and Stamatoyannopoulos, G. (1990). Developmental regulation of human fetal-to-adult globin gene switching in transgenic mice. *Nature* 344, 309-13.
- Epner, E., Reik, A., Cimbor, D., Telling, A., Bender, M. A., Fiering, S., Enver, T., Martin, D. I., Kennedy, M., Keller, G., and Groudine, M. (1998). The beta-globin LCR is not necessary for an open chromatin structure or developmentally regulated transcription of the native mouse beta-globin locus. *Mol Cell* 2, 447-55.
- Evans, T., and Felsenfeld, G. (1989). The erythroid-specific transcription factor Eryf1: a new finger protein. *Cell* 58, 877-85.
- Evans, T., and Felsenfeld, G. (1991). trans-Activation of a globin promoter in nonerythroid cells. *Mol Cell Biol* 11, 843-53.
- Fiering, S., Epner, E., Robinson, K., Zhuang, Y., Telling, A., Hu, M., Martin, D. I., Enver, T., Ley, T. J., and Groudine, M. (1995). Targeted deletion of 5'HS2 of the murine beta-globin LCR reveals that it is not essential for proper regulation of the beta-globin locus. *Genes Dev* 9, 2203-13.
- Follenzi, A., Ailles, L. E., Bakovic, S., Geuna, M., and Naldini, L. (2000). Gene transfer by lentiviral vectors is limited by nuclear translocation and rescued by HIV-1 pol sequences. *Nat Genet* 25, 217-22.
- Forrester, W. C., Takegawa, S., Papayannopoulou, T., Stamatoyannopoulos, G., and Groudine, M. (1987). Evidence for a locus activation region: the formation of developmentally stable hypersensitive sites in globin-expressing hybrids. *Nucleic Acids Res* 15, 10159-77.
- Fouchier, R. A., Simon, J. H., Jaffe, A. B., and Malim, M. H. (1996). Human immunodeficiency virus type 1 Vif does not influence expression or virion incorporation of gag-, pol-, and env-encoded proteins. *J Virol* 70, 8263-9.
- Fox, A. H., Liew, C., Holmes, M., Kowalski, K., Mackay, J., and Crossley, M. (1999). Transcriptional cofactors of the FOG family interact with GATA proteins by means of multiple zinc fingers. *Embo J* 18, 2812-22.
- Francastel, C., Schubeler, D., Martin, D. I., and Groudine, M. (2000). Nuclear compartmentalization and gene activity. *Nat Rev Mol Cell Biol* 1, 137-43.
- Francastel, C., Walters, M. C., Groudine, M., and Martin, D. I. (1999). A functional enhancer suppresses silencing of a transgene and prevents its localization close to centromeric heterochromatin. *Cell* 99, 259-69.
- Fraser, P., Pruzina, S., Antoniou, M., and Grosveld, F. (1993). Each hypersensitive site of the human beta-globin locus control region confers a different developmental pattern of expression on the globin genes. *Genes Dev* 7, 106-13.
- Fraser, P. J., and Curtis, P. J. (1987). Specific pattern of gene expression during induction of mouse erythroleukemia cells. *Genes Dev* 1, 855-61.

- Fujiwara, Y., Browne, C. P., Cunniff, K., Goff, S. C., and Orkin, S. H. (1996). Arrested development of embryonic red cell precursors in mouse embryos lacking transcription factor GATA-1. *Proc Natl Acad Sci U S A* 93, 12355-8.
- Fung, Y. K., Fadly, A. M., Crittenden, L. B., and Kung, H. J. (1981). On the mechanism of retrovirus-induced avian lymphoid leukosis: deletion and integration of the proviruses. *Proc Natl Acad Sci U S A* 78, 3418-22.
- Gabuzda, D. H., Lawrence, K., Langhoff, E., Terwilliger, E., Dorfman, T., Haseltine, W. A., and Sodroski, J. (1992). Role of vif in replication of human immunodeficiency virus type 1 in CD4+ T lymphocytes. *J Virol* 66, 6489-95.
- Gallay, P., Stitt, V., Mundy, C., Oettinger, M., and Trono, D. (1996). Role of the karyopherin pathway in human immunodeficiency virus type 1 nuclear import. *J Virol* 70, 1027-32.
- Gallay, P., Swingler, S., Aiken, C., and Trono, D. (1995). HIV-1 infection of nondividing cells: C-terminal tyrosine phosphorylation of the viral matrix protein is a key regulator. *Cell* 80, 379-88.
- Gaziev, J., and Lucarelli, G. (2003). Stem cell transplantation for hemoglobinopathies. *Curr Opin Pediatr* 15, 24-31.
- Georgopoulos, K., Bigby, M., Wang, J. H., Molnar, A., Wu, P., Winandy, S., and Sharpe, A. (1994). The Ikaros gene is required for the development of all lymphoid lineages. *Cell* 79, 143-56.
- Giampaolo, A., Sterpetti, P., Bulgarini, D., Samoggia, P., Pelosi, E., Valtieri, M., and Peschle, C. (1994). Key functional role and lineage-specific expression of selected HOXB genes in purified hematopoietic progenitor differentiation. *Blood* 84, 3637-47.
- Giardini, C., and Lucarelli, G. (1994). Bone marrow transplantation in the treatment of thalassemia. *Curr Opin Hematol* 1, 170-6.
- Goff, J. P., Shields, D. S., and Greenberger, J. S. (1998). Influence of cytokines on the growth kinetics and immunophenotype of daughter cells resulting from the first division of single CD34(+)Thy-1(+)lin- cells. *Blood* 92, 4098-107.
- Goldsmith, M. A., Warmerdam, M. T., Atchison, R. E., Miller, M. D., and Greene, W. C. (1995). Dissociation of the CD4 downregulation and viral infectivity enhancement functions of human immunodeficiency virus type 1 Nef. *J Virol* 69, 4112-21.
- Gong, Q. H., McDowell, J. C., and Dean, A. (1996). Essential role of NF-E2 in remodeling of chromatin structure and transcriptional activation of the epsilon-globin gene in vivo by 5' hypersensitive site 2 of the beta-globin locus control region. *Mol Cell Biol* 16, 6055-64.
- Goodell, M. A., Brose, K., Paradis, G., Conner, A. S., and Mulligan, R. C. (1996). Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. *J Exp Med* 183, 1797-806.

- Grande, A., Piovani, B., Aiuti, A., Ottolenghi, S., Mavilio, F., and Ferrari, G. (1999). Transcriptional targeting of retroviral vectors to the erythroblastic progeny of transduced hematopoietic stem cells. *Blood* 93, 3276-85.
- Gribnau, J., Diderich, K., Pruzina, S., Calzolari, R., and Fraser, P. (2000). Intergenic transcription and developmental remodeling of chromatin subdomains in the human beta-globin locus. *Mol Cell* 5, 377-86.
- Grosveld, F. (1999). Activation by locus control regions? *Curr Opin Genet Dev* 9, 152-7.
- Grosveld, F., van Assendelft, G. B., Greaves, D. R., and Kollias, G. (1987). Position-independent, high-level expression of the human beta-globin gene in transgenic mice. *Cell* 51, 975-85.
- Hanscombe, O., Whyatt, D., Fraser, P., Yannoutsos, N., Greaves, D., Dillon, N., and Grosveld, F. (1991). Importance of globin gene order for correct developmental expression. *Genes Dev* 5, 1387-94.
- Hao, Q. L., Smogorzewska, E. M., Barsky, L. W., and Crooks, G. M. (1998). In vitro identification of single CD34⁺CD38⁻ cells with both lymphoid and myeloid potential. *Blood* 91, 4145-51.
- Hardison, R., Slightom, J. L., Gumucio, D. L., Goodman, M., Stojanovic, N., and Miller, W. (1997). Locus control regions of mammalian beta-globin gene clusters: combining phylogenetic analyses and experimental results to gain functional insights. *Gene* 205, 73-94.
- Harrich, D., Ulich, C., Garcia-Martinez, L. F., and Gaynor, R. B. (1997). Tat is required for efficient HIV-1 reverse transcription. *Embo J* 16, 1224-35.
- Hartzog, G. A., and Myers, R. M. (1993). Discrimination among potential activators of the beta-globin CACCC element by correlation of binding and transcriptional properties. *Mol Cell Biol* 13, 44-56.
- Haylock, D. N., Horsfall, M. J., Dowse, T. L., Ramshaw, H. S., Niutta, S., Protosaltis, S., Peng, L., Burrell, C., Rappold, I., Buhring, H. J., and Simmons, P. J. (1997). Increased recruitment of hematopoietic progenitor cells underlies the ex vivo expansion potential of FLT3 ligand. *Blood* 90, 2260-72.
- Heinzinger, N. K., Bukinsky, M. I., Haggerty, S. A., Ragland, A. M., Kewalramani, V., Lee, M. A., Gendelman, H. E., Ratner, L., Stevenson, M., and Emerman, M. (1994). The Vpr protein of human immunodeficiency virus type 1 influences nuclear localization of viral nucleic acids in nondividing host cells. *Proc Natl Acad Sci U S A* 91, 7311-5.
- Heuchel, R., Matthias, P., and Schaffner, W. (1989). Two closely spaced promoters are equally activated by a remote enhancer: evidence against a scanning model for enhancer action. *Nucleic Acids Res* 17, 8931-47.
- Higgs, D. R. (1998). Do LCRs open chromatin domains? *Cell* 95, 299-302.

- Howard, J. C., Yousefi, S., Cheong, G., Bernstein, A., and Ben-David, Y. (1993). Temporal order and functional analysis of mutations within the Fli-1 and p53 genes during the erythroleukemias induced by F-MuLV. *Oncogene* 8, 2721-9.
- Hu, M., Krause, D., Greaves, M., Sharkis, S., Dexter, M., Heyworth, C., and Enver, T. (1997). Multilineage gene expression precedes commitment in the hemopoietic system. *Genes Dev* 11, 774-85.
- Huang, J., and Liang, T. J. (1993). A novel hepatitis B virus (HBV) genetic element with Rev response element-like properties that is essential for expression of HBV gene products. *Mol Cell Biol* 13, 7476-86.
- Huang, M., Orenstein, J. M., Martin, M. A., and Freed, E. O. (1995). p6Gag is required for particle production from full-length human immunodeficiency virus type 1 molecular clones expressing protease. *J Virol* 69, 6810-8.
- Huang, Z. M., and Yen, T. S. (1994). Hepatitis B virus RNA element that facilitates accumulation of surface gene transcripts in the cytoplasm. *J Virol* 68, 3193-9.
- Ilyinskii, P. O., and Desrosiers, R. C. (1998). Identification of a sequence element immediately upstream of the polypurine tract that is essential for replication of simian immunodeficiency virus. *Embo J* 17, 3766-74.
- Ito, E., Toki, T., Ishihara, H., Ohtani, H., Gu, L., Yokoyama, M., Engel, J. D., and Yamamoto, M. (1993). Erythroid transcription factor GATA-1 is abundantly transcribed in mouse testis. *Nature* 362, 466-8.
- Iwakuma, T., Cui, Y., and Chang, L. J. (1999). Self-inactivating lentiviral vectors with U3 and U5 modifications. *Virology* 261, 120-32.
- Johnson, K. D., Christensen, H. M., Zhao, B., and Bresnick, E. H. (2001). Distinct mechanisms control RNA polymerase II recruitment to a tissue-specific locus control region and a downstream promoter. *Mol Cell* 8, 465-71.
- Jordan, C. T., and Lemischka, I. R. (1990). Clonal and systemic analysis of long-term hematopoiesis in the mouse. *Genes Dev* 4, 220-32.
- Jowett, J. B., Planelles, V., Poon, B., Shah, N. P., Chen, M. L., and Chen, I. S. (1995). The human immunodeficiency virus type 1 vpr gene arrests infected T cells in the G2 + M phase of the cell cycle. *J Virol* 69, 6304-13.
- Kafri, T., van Praag, H., Gage, F. H., and Verma, I. M. (2000). Lentiviral vectors: regulated gene expression. *Mol Ther* 1, 516-21.
- Kalberer, C. P., Pawliuk, R., Imren, S., Bachelot, T., Takekoshi, K. J., Fabry, M., Eaves, C. J., London, I. M., Humphries, R. K., and Leboulch, P. (2000). Preselection of retrovirally transduced bone marrow avoids subsequent stem cell gene silencing and age-dependent extinction of expression of human beta-globin in engrafted mice. *Proc Natl Acad Sci U S A* 97, 5411-5.

- Kamel-Reid, S., and Dick, J. E. (1988). Engraftment of immune-deficient mice with human hematopoietic stem cells. *Science* 242, 1706-9.
- Kaptein, L. C., Breuer, M., Valerio, D., and van Beusechem, V. W. (1998). Expression pattern of CD2 locus control region containing retroviral vectors in hemopoietic cells in vitro and in vivo. *Gene Ther* 5, 320-30.
- Karanu, F. N., Murdoch, B., Gallacher, L., Wu, D. M., Koremoto, M., Sakano, S., and Bhatia, M. (2000). The notch ligand jagged-1 represents a novel growth factor of human hematopoietic stem cells. *J Exp Med* 192, 1365-72.
- Karpen, G. H. (1994). Position-effect variegation and the new biology of heterochromatin. *Curr Opin Genet Dev* 4, 281-91.
- Kaushansky, K. (1998). Growth factors and hematopoietic cell fate. A new feature: controversies in hematology. *Blood* 92, 345-4.
- Kaushansky, K. (1998). Thrombopoietin. *N Engl J Med* 339, 746-54.
- Kaushansky, K., Broudy, V. C., Lin, N., Jorgensen, M. J., McCarty, J., Fox, N., Zucker-Franklin, D., and Lofton-Day, C. (1995). Thrombopoietin, the Mp1 ligand, is essential for full megakaryocyte development. *Proc Natl Acad Sci U S A* 92, 3234-8.
- Kay, M. A., Glorioso, J. C., and Naldini, L. (2001). Viral vectors for gene therapy: the art of turning infectious agents into vehicles of therapeutics. *Nat Med* 7, 33-40.
- Keller, G., and Snodgrass, R. (1990). Life span of multipotential hematopoietic stem cells in vivo. *J Exp Med* 171, 1407-18.
- Kerkau, T., Bacik, I., Bennink, J. R., Yewdell, J. W., Hunig, T., Schimpl, A., and Schubert, U. (1997). The human immunodeficiency virus type 1 (HIV-1) Vpu protein interferes with an early step in the biosynthesis of major histocompatibility complex (MHC) class I molecules. *J Exp Med* 185, 1295-305.
- Kestler, H. W., 3rd, Ringler, D. J., Mori, K., Panicali, D. L., Sehgal, P. K., Daniel, M. D., and Desrosiers, R. C. (1991). Importance of the nef gene for maintenance of high virus loads and for development of AIDS. *Cell* 65, 651-62.
- Kim, V. N., Mitrophanous, K., Kingsman, S. M., and Kingsman, A. J. (1998). Minimal requirement for a lentivirus vector based on human immunodeficiency virus type 1. *J Virol* 72, 811-6.
- Kim, Y. K., Bourgeois, C. F., Isel, C., Churcher, M. J., and Karn, J. (2002). Phosphorylation of the RNA polymerase II carboxyl-terminal domain by CDK9 is directly responsible for human immunodeficiency virus type 1 Tat-activated transcriptional elongation. *Mol Cell Biol* 22, 4622-37.
- Kingston, R. E., and Narlikar, G. J. (1999). ATP-dependent remodeling and acetylation as regulators of chromatin fluidity. *Genes Dev* 13, 2339-52.

- Kirby, S., Walton, W., and Smithies, O. (2000). Hematopoietic stem cells with controllable tEpoR transgenes have a competitive advantage in bone marrow transplantation. *Blood* 95, 3710-5.
- Kirby, S. L., Cook, D. N., Walton, W., and Smithies, O. (1996). Proliferation of multipotent hematopoietic cells controlled by a truncated erythropoietin receptor transgene. *Proc Natl Acad Sci U S A* 93, 9402-7.
- Klages, N., Zufferey, R., and Trono, D. (2000). A stable system for the high-titer production of multiply attenuated lentiviral vectors. *Mol Ther* 2, 170-6.
- Klimkait, T., Strebel, K., Hoggan, M. D., Martin, M. A., and Orenstein, J. M. (1990). The human immunodeficiency virus type 1-specific protein vpu is required for efficient virus maturation and release. *J Virol* 64, 621-9.
- Klug, C. A., Morrison, S. J., Masek, M., Hahm, K., Smale, S. T., and Weissman, I. L. (1998). Hematopoietic stem cells and lymphoid progenitors express different Ikaros isoforms, and Ikaros is localized to heterochromatin in immature lymphocytes. *Proc Natl Acad Sci U S A* 95, 657-62.
- Kohn, D. B. (1997). Gene therapy for haematopoietic and lymphoid disorders. *Clin Exp Immunol* 107 Suppl 1, 54-7.
- Kohn, D. B. (1999). Gene therapy using hematopoietic stem cells. *Curr Opin Mol Ther* 1, 437-42.
- Koipally, J., Renold, A., Kim, J., and Georgopoulos, K. (1999). Repression by Ikaros and Aiolos is mediated through histone deacetylase complexes. *Embo J* 18, 3090-100.
- Kondo, M., Scherer, D. C., Miyamoto, T., King, A. G., Akashi, K., Sugamura, K., and Weissman, I. L. (2000). Cell-fate conversion of lymphoid-committed progenitors by instructive actions of cytokines. *Nature* 407, 383-6.
- Kondo, M., Weissman, I. L., and Akashi, K. (1997). Identification of clonogenic common lymphoid progenitors in mouse bone marrow. *Cell* 91, 661-72.
- Kotkow, K. J., and Orkin, S. H. (1996). Complexity of the erythroid transcription factor NF-E2 as revealed by gene targeting of the mouse p18 NF-E2 locus. *Proc Natl Acad Sci U S A* 93, 3514-8.
- Kyoizumi, S., Baum, C. M., Kaneshima, H., McCune, J. M., Yee, E. J., and Namikawa, R. (1992). Implantation and maintenance of functional human bone marrow in SCID-hu mice. *Blood* 79, 1704-11.
- Larochelle, A., Peng, K. W., and Russell, S. J. (2002). Lentiviral vector targeting. *Curr Top Microbiol Immunol* 261, 143-63.
- Larochelle, A., Vormoor, J., Hanenberg, H., Wang, J. C., Bhatia, M., Lapidot, T., Moritz, T., Murdoch, B., Xiao, X. L., Kato, I., Williams, D. A., and Dick, J. E. (1996). Identification of primitive human hematopoietic cells capable of repopulating NOD/SCID mouse bone marrow: implications for gene therapy. *Nat Med* 2, 1329-37.

- Lee, E. Y., Chang, C. Y., Hu, N., Wang, Y. C., Lai, C. C., Herrup, K., Lee, W. H., and Bradley, A. (1992). Mice deficient for Rb are nonviable and show defects in neurogenesis and haematopoiesis. *Nature* 359, 288-94.
- Letting, D. L., Rakowski, C., Weiss, M. J., and Blobel, G. A. (2003). Formation of a tissue-specific histone acetylation pattern by the hematopoietic transcription factor GATA-1. *Mol Cell Biol* 23, 1334-40.
- Levings, P. P., and Bungert, J. (2002). The human beta-globin locus control region. *Eur J Biochem* 269, 1589-99.
- Lewis, B. A., and Orkin, S. H. (1995). A functional initiator element in the human beta-globin promoter. *J Biol Chem* 270, 28139-44.
- Lewis, P. F., and Emerman, M. (1994). Passage through mitosis is required for oncoretroviruses but not for the human immunodeficiency virus. *J Virol* 68, 510-6.
- Li, G., Lim, K. C., Engel, J. D., and Bungert, J. (1998). Individual LCR hypersensitive sites cooperate to generate an open chromatin domain spanning the human beta-globin locus. *Genes Cells* 3, 415-29.
- Lotti, F., Menguzzato, E., Rossi, C., Naldini, L., Ailles, L., Mavilio, F., and Ferrari, G. (2002). Transcriptional targeting of lentiviral vectors by long terminal repeat enhancer replacement. *J Virol* 76, 3996-4007.
- Luban, J., and Goff, S. P. (1994). Mutational analysis of cis-acting packaging signals in human immunodeficiency virus type 1 RNA. *J Virol* 68, 3784-93.
- Lucarelli, G., Andreani, M., and Angelucci, E. (2001). The cure of the thalassemia with bone marrow transplantation. *Bone Marrow Transplant* 28 *Suppl 1*, S11-3.
- Luciw, P. A. (1996). *Human Immunodeficiency Viruses and Their Replication*, 3rd Edition, L.-R. Publishers, ed.
- Lykke-Andersen, J. (2001). mRNA quality control: Marking the message for life or death. *Curr Biol* 11, R88-91.
- Ma, Y., Ramezani, A., Lewis, R., Hawley, R. G., and Thomson, J. A. (2003). High-level sustained transgene expression in human embryonic stem cells using lentiviral vectors. *Stem Cells* 21, 111-7.
- Madani, N., and Kabat, D. (1998). An endogenous inhibitor of human immunodeficiency virus in human lymphocytes is overcome by the viral Vif protein. *J Virol* 72, 10251-5.
- Maeno, M., Mead, P. E., Kelley, C., Xu, R. H., Kung, H. F., Suzuki, A., Ueno, N., and Zon, L. I. (1996). The role of BMP-4 and GATA-2 in the induction and differentiation of hematopoietic mesoderm in *Xenopus laevis*. *Blood* 88, 1965-72.

- Mahmoudi, T., Katsani, K. R., and Verrijzer, C. P. (2002). GAGA can mediate enhancer function in trans by linking two separate DNA molecules. *Embo J* 21, 1775-81.
- Manaia, A., Lemarchandel, V., Klaine, M., Max-Audit, I., Romeo, P., Dieterlen-Lievre, F., and Godin, I. (2000). Lmo2 and GATA-3 associated expression in intraembryonic hemogenic sites. *Development* 127, 643-53.
- Marodon, G., Mouly, E., Blair, E. J., Frisen, C., Lemoine, F. M., and Klatzmann, D. (2003). Specific transgene expression in human and mouse CD4+ cells using lentiviral vectors with regulatory sequences from the CD4 gene. *Blood* 101, 3416-23.
- Marshall, C. J., Kinnon, C., and Thrasher, A. J. (2000). Polarized expression of bone morphogenetic protein-4 in the human aorta- gonad-mesonephros region. *Blood* 96, 1591-3.
- Martin, D. I. (2001). Transcriptional enhancers--on/off gene regulation as an adaptation to silencing in higher eukaryotic nuclei. *Trends Genet* 17, 444-8.
- Martin, D. I., Fiering, S., and Groudine, M. (1996). Regulation of beta-globin gene expression: straightening out the locus. *Curr Opin Genet Dev* 6, 488-95.
- Martin, F., Chowdhury, S., Neil, S., Phillipps, N., and Collins, M. K. (2002). Envelope-targeted retrovirus vectors transduce melanoma xenografts but not spleen or liver. *Mol Ther* 5, 269-74.
- Martin, P., and Papayannopoulou, T. (1982). HEL cells: a new human erythroleukemia cell line with spontaneous and induced globin expression. *Science* 216, 1233-5.
- May, C., Rivella, S., Callegari, J., Heller, G., Gaensler, K. M., Luzzatto, L., and Sadelain, M. (2000). Therapeutic haemoglobin synthesis in beta-thalassaemic mice expressing lentivirus-encoded human beta-globin. *Nature* 406, 82-6.
- May, C., Rivella, S., Chadburn, A., and Sadelain, M. (2002). Successful treatment of murine beta-thalassemia intermedia by transfer of the human beta-globin gene. *Blood* 99, 1902-8.
- May, C., and Sadelain, M. (2001). A promising genetic approach to the treatment of beta-thalassemia. *Trends Cardiovasc Med* 11, 276-80.
- McCune, J. M., Namikawa, R., Kaneshima, H., Shultz, L. D., Lieberman, M., and Weissman, I. L. (1988). The SCID-hu mouse: murine model for the analysis of human hematolymphoid differentiation and function. *Science* 241, 1632-9.
- Medvinsky, A., and Dzierzak, E. (1996). Definitive hematopoiesis is autonomously initiated by the AGM region. *Cell* 86, 897-906.
- Metcalf, D., and Burgess, A. W. (1982). Clonal analysis of progenitor cell commitment of granulocyte or macrophage production. *J Cell Physiol* 111, 275-83.

- Mignotte, V., Eleouet, J. F., Raich, N., and Romeo, P. H. (1989). Cis- and trans-acting elements involved in the regulation of the erythroid promoter of the human porphobilinogen deaminase gene. *Proc Natl Acad Sci U S A* 86, 6548-52.
- Mikkola, H. K., Klintman, J., Yang, H., Hock, H., Schlaeger, T. M., Fujiwara, Y., and Orkin, S. H. (2003). Haematopoietic stem cells retain long-term repopulating activity and multipotency in the absence of stem-cell leukaemia SCL/tal-1 gene. *Nature* 421, 547-51.
- Miller, C. L., and Eaves, C. J. (1997). Expansion in vitro of adult murine hematopoietic stem cells with transplantable lympho-myeloid reconstituting ability. *Proc Natl Acad Sci U S A* 94, 13648-53.
- Miller, J. S., McCullar, V., Punzel, M., Lemischka, I. R., and Moore, K. A. (1999). Single adult human CD34(+)/Lin-/CD38(-) progenitors give rise to natural killer cells, B-lineage cells, dendritic cells, and myeloid cells. *Blood* 93, 96-106.
- Milot, E., Strouboulis, J., Trimborn, T., Wijgerde, M., de Boer, E., Langeveld, A., Tan-Un, K., Vergeer, W., Yannoutsos, N., Grosveld, F., and Fraser, P. (1996). Heterochromatin effects on the frequency and duration of LCR-mediated gene transcription. *Cell* 87, 105-14.
- Miyoshi, H., Blomer, U., Takahashi, M., Gage, F. H., and Verma, I. M. (1998). Development of a self-inactivating lentivirus vector. *J Virol* 72, 8150-7.
- Miyoshi, H., Smith, K. A., Mosier, D. E., Verma, I. M., and Torbett, B. E. (1999). Transduction of human CD34+ cells that mediate long-term engraftment of NOD/SCID mice by HIV vectors. *Science* 283, 682-6.
- Moreau-Gaudry, F., Xia, P., Jiang, G., Perelman, N. P., Bauer, G., Ellis, J., Surinya, K. H., Mavilio, F., Shen, C. K., and Malik, P. (2001). High-level erythroid-specific gene expression in primary human and murine hematopoietic cells with self-inactivating lentiviral vectors. *Blood* 98, 2664-72.
- Morrison, S. J., Uchida, N., and Weissman, I. L. (1995). The biology of hematopoietic stem cells. *Annu Rev Cell Dev Biol* 11, 35-71.
- Morrison, S. J., Wandycz, A. M., Akashi, K., Globerson, A., and Weissman, I. L. (1996). The aging of hematopoietic stem cells. *Nat Med* 2, 1011-6.
- Morrison, S. J., Wandycz, A. M., Hemmati, H. D., Wright, D. E., and Weissman, I. L. (1997). Identification of a lineage of multipotent hematopoietic progenitors. *Development* 124, 1929-39.
- Morrison, S. J., and Weissman, I. L. (1994). The long-term repopulating subset of hematopoietic stem cells is deterministic and isolatable by phenotype. *Immunity* 1, 661-73.
- Motohashi, H., Shavit, J. A., Igarashi, K., Yamamoto, M., and Engel, J. D. (1997). The world according to Maf. *Nucleic Acids Res* 25, 2953-59.

- Muller-Sieburg, C. E., and Deryugina, E. (1995). The stromal cells' guide to the stem cell universe. *Stem Cells* 13, 477-86.
- Murray, L., Chen, B., Galy, A., Chen, S., Tushinski, R., Uchida, N., Negrin, R., Tricot, G., Jagannath, S., Vesole, D., and et al. (1995). Enrichment of human hematopoietic stem cell activity in the CD34+Thy- 1+Lin- subpopulation from mobilized peripheral blood. *Blood* 85, 368-78.
- Murray, L., DiGiusto, D., Chen, B., Chen, S., Combs, J., Conti, A., Galy, A., Negrin, R., Tricot, G., and Tsukamoto, A. (1994). Analysis of human hematopoietic stem cell populations. *Blood Cells* 20, 364-9; discussion 369-70.
- Murray, L., Travis, M., Luens-Abitorabi, K., Olsson, K., Plavec, I., Forestell, S., Hanania, E. G., and Hill, B. (2000). Addition of the human interferon beta scaffold attachment region to retroviral vector backbones increases the level of in vivo transgene expression among progeny of engrafted human hematopoietic stem cells. *Hum Gene Ther* 11, 2039-50.
- Murray, L. J., Young, J. C., Osborne, L. J., Luens, K. M., Scollay, R., and Hill, B. L. (1999). Thrombopoietin, flt3, and kit ligands together suppress apoptosis of human mobilized CD34+ cells and recruit primitive CD34+ Thy-1+ cells into rapid division. *Exp Hematol* 27, 1019-28.
- Myers, R. M., Cowie, A., Stuve, L., Hartzog, G., and Gaensler, K. (1989). Genetic and biochemical analysis of the mouse beta-major globin promoter. *Prog Clin Biol Res* 316A, 117-27.
- Naldini, L., Blomer, U., Gally, P., Ory, D., Mulligan, R., Gage, F. H., Verma, I. M., and Trono, D. (1996). In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. *Science* 272, 263-7.
- Namciu, S. J., Blochlinger, K. B., and Fournier, R. E. (1998). Human matrix attachment regions insulate transgene expression from chromosomal position effects in *Drosophila melanogaster*. *Mol Cell Biol* 18, 2382-91.
- Nerlov, C., Querfurth, E., Kulesa, H., and Graf, T. (2000). GATA-1 interacts with the myeloid PU.1 transcription factor and represses PU.1-dependent transcription. *Blood* 95, 2543-51.
- Ney, P. A., Sorrentino, B. P., Lowrey, C. H., and Nienhuis, A. W. (1990). Inducibility of the HS II enhancer depends on binding of an erythroid specific nuclear protein. *Nucleic Acids Res* 18, 6011-7.
- Nichols, K. E., Crispino, J. D., Poncz, M., White, J. G., Orkin, S. H., Maris, J. M., and Weiss, M. J. (2000). Familial dyserythropoietic anaemia and thrombocytopenia due to an inherited mutation in GATA1. *Nat Genet* 24, 266-70.
- Nicolis, S., Bertini, C., Ronchi, A., Crotta, S., Lanfranco, L., Moroni, E., Giglioni, B., and Ottolenghi, S. (1991). An erythroid specific enhancer upstream to the gene encoding the cell- type specific transcription factor GATA-1. *Nucleic Acids Res* 19, 5285-91.

- Nienhuis, A. W., and Stamatoyannopoulos, G. (1978). Hemoglobin switching. *Cell* 15, 307-15.
- Nolta, J. A., Dao, M. A., Wells, S., Smogorzewska, E. M., and Kohn, D. B. (1996). Transduction of pluripotent human hematopoietic stem cells demonstrated by clonal analysis after engraftment in immune-deficient mice. *Proc Natl Acad Sci U S A* 93, 2414-9.
- Ogawa, K., Shibata, R., Kiyomasu, T., Higuchi, I., Kishida, Y., Ishimoto, A., and Adachi, A. (1989). Mutational analysis of the human immunodeficiency virus vpr open reading frame. *J Virol* 63, 4110-4.
- Ogawa, M. (1993). Differentiation and proliferation of hematopoietic stem cells. *Blood* 81, 2844-53.
- Ogawa, M. (1999). Stochastic model revisited. *Int J Hematol* 69, 2-5.
- Okuda, T., van Deursen, J., Hiebert, S. W., Grosveld, G., and Downing, J. R. (1996). AML1, the target of multiple chromosomal translocations in human leukemia, is essential for normal fetal liver hematopoiesis. *Cell* 84, 321-30.
- Olivieri, N. F. (1999). The beta-thalassemias. *N Engl J Med* 341, 99-109.
- Olivieri, N. F., Brittenham, G. M., McLaren, C. E., Templeton, D. M., Cameron, R. G., McClelland, R. A., Burt, A. D., and Fleming, K. A. (1998). Long-term safety and effectiveness of iron-chelation therapy with deferiprone for thalassemia major. *N Engl J Med* 339, 417-23.
- Orkin, S. H. (1992). GATA-binding transcription factors in hematopoietic cells. *Blood* 80, 575-81.
- Orkin, S. H. (1995). Regulation of globin gene expression in erythroid cells. *Eur J Biochem* 231, 271-81.
- Orkin, S. H., and Zon, L. I. (1997). Genetics of erythropoiesis: induced mutations in mice and zebrafish. *Annu Rev Genet* 31, 33-60.
- Otto, E., Jones-Trower, A., Vanin, E. F., Stambaugh, K., Mueller, S. N., Anderson, W. F., and McGarrity, G. J. (1994). Characterization of a replication-competent retrovirus resulting from recombination of packaging and vector sequences. *Hum Gene Ther* 5, 567-75.
- Palis, J., Robertson, S., Kennedy, M., Wall, C., and Keller, G. (1999). Development of erythroid and myeloid progenitors in the yolk sac and embryo proper of the mouse. *Development* 126, 5073-84.
- Papayannopoulou, T., Nakamoto, B., Kurachi, S., and Nelson, R. (1987). Analysis of the erythroid phenotype of HEL cells: clonal variation and the effect of inducers. *Blood* 70, 1764-72.

- Park, F., Ohashi, K., Chiu, W., Naldini, L., and Kay, M. A. (2000). Efficient lentiviral transduction of liver requires cell cycling in vivo. *Nat Genet* 24, 49-52.
java/Propub/genetics/ng0100_49.fulltext java/Propub/genetics/ng0100_49.abstract.
- Peault, B., Weissman, I. L., Baum, C., McCune, J. M., and Tsukamoto, A. (1991). Lymphoid reconstitution of the human fetal thymus in SCID mice with CD34+ precursor cells. *J Exp Med* 174, 1283-6.
- Peng, K. W., Vile, R., Cosset, F. L., and Russell, S. (1999). Selective transduction of protease-rich tumors by matrix-metalloproteinase-targeted retroviral vectors. *Gene Ther* 6, 1552-7.
- Persons, D. A., Allay, E. R., Sabatino, D. E., Kelly, P., Bodine, D. M., and Nienhuis, A. W. (2001). Functional requirements for phenotypic correction of murine beta-thalassemia: implications for human gene therapy. *Blood* 97, 3275-82.
- Peterson, C. L., and Logie, C. (2000). Recruitment of chromatin remodeling machines. *J Cell Biochem* 78, 179-85.
- Pevny, L., Simon, M. C., Robertson, E., Klein, W. H., Tsai, S. F., D'Agati, V., Orkin, S. H., and Costantini, F. (1991). Erythroid differentiation in chimaeric mice blocked by a targeted mutation in the gene for transcription factor GATA-1. *Nature* 349, 257-60.
- Philipsen, S., Pruzina, S., and Grosveld, F. (1993). The minimal requirements for activity in transgenic mice of hypersensitive site 3 of the beta globin locus control region. *Embo J* 12, 1077-85.
- Piguet, V., Gu, F., Foti, M., Demarex, N., Gruenberg, J., Carpentier, J. L., and Trono, D. (1999). Nef-induced CD4 degradation: a diacidic-based motif in Nef functions as a lysosomal targeting signal through the binding of beta-COP in endosomes. *Cell* 97, 63-73.
- Poon, B., Grovit-Ferbas, K., Stewart, S. A., and Chen, I. S. (1998). Cell cycle arrest by Vpr in HIV-1 virions and insensitivity to antiretroviral agents. *Science* 281, 266-9.
- Prioleau, M. N., Nony, P., Simpson, M., and Felsenfeld, G. (1999). An insulator element and condensed chromatin region separate the chicken beta-globin locus from an independently regulated erythroid-specific folate receptor gene. *Embo J* 18, 4035-48.
- Purcell, D. F., Brosius, C. M., Vanin, E. F., Buckler, C. E., Nienhuis, A. W., and Martin, M. A. (1996). An array of murine leukemia virus-related elements is transmitted and expressed in a primate recipient of retroviral gene transfer. *J Virol* 70, 887-97.
- Rabbitts, T. H. (1994). Chromosomal translocations in human cancer. *Nature* 372, 143-9.
- Raftopoulos, H., Ward, M., Leboulch, P., and Bank, A. (1997). Long-term transfer and expression of the human beta-globin gene in a mouse transplant model. *Blood* 90, 3414-22.

- Ramezani, A., Hawley, T. S., and Hawley, R. G. (2003). Performance- and safety-enhanced lentiviral vectors containing the human interferon- β scaffold attachment region and the chicken β -globin insulator. *Blood*.
- Reik, A., Telling, A., Zitnik, G., Cimborra, D., Epner, E., and Groudine, M. (1998). The locus control region is necessary for gene expression in the human β -globin locus but not the maintenance of an open chromatin structure in erythroid cells. *Mol Cell Biol* 18, 5992-6000.
- Rekhtman, N., Radparvar, F., Evans, T., and Skoultschi, A. I. (1999). Direct interaction of hematopoietic transcription factors PU.1 and GATA-1: functional antagonism in erythroid cells. *Genes Dev* 13, 1398-411.
- Richard, E., Mendez, M., Mazurier, F., Morel, C., Costet, P., Xia, P., Fontanellas, A., Geronimi, F., Cario-Andre, M., Taine, L., Ged, C., Malik, P., de Verneuil, H., and Moreau-Gaudry, F. (2001). Gene therapy of a mouse model of protoporphyria with a self-inactivating erythroid-specific lentiviral vector without preselection. *Mol Ther* 4, 331-8.
- Richter, J. (1997). Gene transfer to hematopoietic cells--the clinical experience. *Eur J Haematol* 59, 67-75.
- Rivella, S., Callegari, J. A., May, C., Tan, C. W., and Sadelain, M. (2000). The cHS4 insulator increases the probability of retroviral expression at random chromosomal integration sites. *J Virol* 74, 4679-87.
- Rivella, S., May, C., Chadburn, A., Riviere, I., and Sadelain, M. (2003). A novel murine model of Cooley anemia and its rescue by lentiviral-mediated human β -globin gene transfer. *Blood* 101, 2932-9.
- Rivella, S., and Sadelain, M. (1998). Genetic treatment of severe hemoglobinopathies: the combat against transgene variegation and transgene silencing. *Semin Hematol* 35, 112-25.
- Robb, L., Lyons, I., Li, R., Hartley, L., Kontgen, F., Harvey, R. P., Metcalf, D., and Begley, C. G. (1995). Absence of yolk sac hematopoiesis from mice with a targeted disruption of the *scl* gene. *Proc Natl Acad Sci U S A* 92, 7075-9.
- Rothenberg, E. V. (2000). Stepwise specification of lymphocyte developmental lineages. *Curr Opin Genet Dev* 10, 370-9.
- Russell, D. W., and Kay, M. A. (1999). Adeno-associated virus vectors and hematology. *Blood* 94, 864-74.
- Sabatino, D. E., Seidel, N. E., Aviles-Mendoza, G. J., Cline, A. P., Anderson, S. M., Gallagher, P. G., and Bodine, D. M. (2000). Long-term expression of gamma-globin mRNA in mouse erythrocytes from retrovirus vectors containing the human gamma-globin gene fused to the ankyrin-1 promoter. *Proc Natl Acad Sci U S A* 97, 13294-9.
- Sabatino, D. E., Wong, C., Cline, A. P., Pyle, L., Garrett, L. J., Gallagher, P. G., and Bodine, D. M. (2000). A minimal ankyrin promoter linked to a human gamma-globin

- gene demonstrates erythroid specific copy number dependent expression with minimal position or enhancer dependence in transgenic mice. *J Biol Chem* 275, 28549-54.
- Sabbattini, P., Lundgren, M., Georgiou, A., Chow, C., Warnes, G., and Dillon, N. (2001). Binding of Ikaros to the lambda5 promoter silences transcription through a mechanism that does not require heterochromatin formation. *Embo J* 20, 2812-22.
- Sadelain, M. (2002). Globin gene transfer for the treatment of severe hemoglobinopathies: a paradigm for stem cell-based gene therapy. *J Gene Med* 4, 113-21.
- Sadelain, M., Frassoni, F., and Riviere, I. (2000). Issues in the manufacture and transplantation of genetically modified hematopoietic stem cells. *Curr Opin Hematol* 7, 364-77.
- Sadelain, M., Wang, C. H., Antoniou, M., Grosveld, F., and Mulligan, R. C. (1995). Generation of a high-titer retroviral vector capable of expressing high levels of the human beta-globin gene. *Proc Natl Acad Sci U S A* 92, 6728-32.
- Sakai, H., Kawamura, M., Sakuragi, J., Sakuragi, S., Shibata, R., Ishimoto, A., Ono, N., Ueda, S., and Adachi, A. (1993). Integration is essential for efficient gene expression of human immunodeficiency virus type 1. *J Virol* 67, 1169-74.
- Sakai, H., Shibata, R., Sakuragi, J., Sakuragi, S., Kawamura, M., and Adachi, A. (1993). Cell-dependent requirement of human immunodeficiency virus type 1 Vif protein for maturation of virus particles. *J Virol* 67, 1663-6.
- Sauvageau, G., Lansdorp, P. M., Eaves, C. J., Hogge, D. E., Dragowska, W. H., Reid, D. S., Largman, C., Lawrence, H. J., and Humphries, R. K. (1994). Differential expression of homeobox genes in functionally distinct CD34+ subpopulations of human bone marrow cells. *Proc Natl Acad Sci U S A* 91, 12223-7.
- Sauvageau, G., Thorsteinsdottir, U., Eaves, C. J., Lawrence, H. J., Largman, C., Lansdorp, P. M., and Humphries, R. K. (1995). Overexpression of HOXB4 in hematopoietic cells causes the selective expansion of more primitive populations in vitro and in vivo. *Genes Dev* 9, 1753-65.
- Sawado, T., Halow, J., Bender, M. A., and Groudine, M. (2003). The beta -globin locus control region (LCR) functions primarily by enhancing the transition from transcription initiation to elongation. *Genes Dev*.
- Schubeler, D., Francastel, C., Cimbora, D. M., Reik, A., Martin, D. I., and Groudine, M. (2000). Nuclear localization and histone acetylation: a pathway for chromatin opening and transcriptional activation of the human beta-globin locus. *Genes Dev* 14, 940-50.
- Schubeler, D., Mielke, C., Maass, K., and Bode, J. (1996). Scaffold/matrix-attached regions act upon transcription in a context-dependent manner. *Biochemistry* 35, 11160-9.

- Scott, E. W., Simon, M. C., Anastasi, J., and Singh, H. (1994). Requirement of transcription factor PU.1 in the development of multiple hematopoietic lineages. *Science* 265, 1573-7.
- Scott, M. P. (1992). Vertebrate homeobox gene nomenclature. *Cell* 71, 551-3.
- Selig, L., Pages, J. C., Tanchou, V., Preveral, S., Berlioz-Torrent, C., Liu, L. X., Erdtmann, L., Darlix, J., Benarous, R., and Benichou, S. (1999). Interaction with the p6 domain of the gag precursor mediates incorporation into virions of Vpr and Vpx proteins from primate lentiviruses. *J Virol* 73, 592-600.
- Shimizu, R., Takahashi, S., Ohneda, K., Engel, J. D., and Yamamoto, M. (2001). In vivo requirements for GATA-1 functional domains during primitive and definitive erythropoiesis. *Embo J* 20, 5250-60.
- Shivdasani, R. A., Fujiwara, Y., McDevitt, M. A., and Orkin, S. H. (1997). A lineage-selective knockout establishes the critical role of transcription factor GATA-1 in megakaryocyte growth and platelet development. *Embo J* 16, 3965-73.
- Shivdasani, R. A., and Orkin, S. H. (1996). The transcriptional control of hematopoiesis. *Blood* 87, 4025-39.
- Singer, D., Cooper, M., Maniatis, G. M., Marks, P. A., and Rifkind, R. A. (1974). Erythropoietic differentiation in colonies of cells transformed by Friend virus. *Proc Natl Acad Sci U S A* 71, 2668-70.
- Smith, L. G., Weissman, I. L., and Heimfeld, S. (1991). Clonal analysis of hematopoietic stem-cell differentiation in vivo. *Proc Natl Acad Sci U S A* 88, 2788-92.
- Spangrude, G. J., Heimfeld, S., and Weissman, I. L. (1988). Purification and characterization of mouse hematopoietic stem cells. *Science* 241, 58-62.
- Sposi, N. M., Zon, L. I., Care, A., Valtieri, M., Testa, U., Gabbianelli, M., Mariani, G., Bottero, L., Mather, C., Orkin, S. H., and et al. (1992). Cell cycle-dependent initiation and lineage-dependent abrogation of GATA-1 expression in pure differentiating hematopoietic progenitors. *Proc Natl Acad Sci U S A* 89, 6353-7.
- Srinivasakumar, N., and Schuening, F. G. (1999). A lentivirus packaging system based on alternative RNA transport mechanisms to express helper and gene transfer vector RNAs and its use to study the requirement of accessory proteins for particle formation and gene delivery. *J Virol* 73, 9589-98.
- Stevens, A., Wang, Y., Bremer, K., Zhang, J., Hoepfner, R., Antoniou, M., Schoenberg, D. R., and Maquat, L. E. (2002). Beta -Globin mRNA decay in erythroid cells: UG site-preferred endonucleolytic cleavage that is augmented by a premature termination codon. *Proc Natl Acad Sci U S A* 99, 12741-6.
- Stewart, S. A., Poon, B., Jowett, J. B., and Chen, I. S. (1997). Human immunodeficiency virus type 1 Vpr induces apoptosis following cell cycle arrest. *J Virol* 71, 5579-92.

- Stuve, L. L., and Myers, R. M. (1990). A directly repeated sequence in the beta-globin promoter regulates transcription in murine erythroleukemia cells. *Mol Cell Biol* *10*, 972-81.
- Sutherland, H. G., Martin, D. I., and Whitelaw, E. (1997). A globin enhancer acts by increasing the proportion of erythrocytes expressing a linked transgene. *Mol Cell Biol* *17*, 1607-14.
- Sutherland, H. J., Lansdorp, P. M., Henkelman, D. H., Eaves, A. C., and Eaves, C. J. (1990). Functional characterization of individual human hematopoietic stem cells cultured at limiting dilution on supportive marrow stromal layers. *Proc Natl Acad Sci U S A* *87*, 3584-8.
- Sutter, N. B., Scalzo, D., Fiering, S., Groudine, M., and Martin, D. I. (2003). Chromatin insulation by a transcriptional activator. *Proc Natl Acad Sci U S A* *100*, 1105-10.
- Talbot, D., Collis, P., Antoniou, M., Vidal, M., Grosveld, F., and Greaves, D. R. (1989). A dominant control region from the human beta-globin locus conferring integration site-independent gene expression. *Nature* *338*, 352-5.
- Talbot, D., and Grosveld, F. (1991). The 5'HS2 of the globin locus control region enhances transcription through the interaction of a multimeric complex binding at two functionally distinct NF-E2 binding sites. *Embo J* *10*, 1391-8.
- Tamir, A., Howard, J., Higgins, R. R., Li, Y. J., Berger, L., Zacksenhaus, E., Reis, M., and Ben-David, Y. (1999). Fli-1, an Ets-related transcription factor, regulates erythropoietin-induced erythroid proliferation and differentiation: evidence for direct transcriptional repression of the Rb gene during differentiation. *Mol Cell Biol* *19*, 4452-64.
- Tang, Y., and Qian, R. L. (1998). [Studies on DNA-protein interactions in the HS3 of the human beta-LCR]. *Shi Yan Sheng Wu Xue Bao* *31*, 179-86.
- Tanimoto, K., Liu, Q., Bungert, J., and Engel, J. D. (1999). Effects of altered gene order or orientation of the locus control region on human beta-globin gene expression in mice. *Nature* *398*, 344-8.
- Tevosian, S. G., Deconinck, A. E., Cantor, A. B., Rieff, H. I., Fujiwara, Y., Corfas, G., and Orkin, S. H. (1999). FOG-2: A novel GATA-family cofactor related to multitype zinc-finger proteins Friend of GATA-1 and U-shaped. *Proc Natl Acad Sci U S A* *96*, 950-5.
- Tevosian, S. G., Deconinck, A. E., Tanaka, M., Schinke, M., Litovsky, S. H., Izumo, S., Fujiwara, Y., and Orkin, S. H. (2000). FOG-2, a cofactor for GATA transcription factors, is essential for heart morphogenesis and development of coronary vessels from epicardium. *Cell* *101*, 729-39.
- Thorsteinsdottir, U., Sauvageau, G., and Humphries, R. K. (1999). Enhanced in vivo regenerative potential of HOXB4-transduced hematopoietic stem cells with regulation of their pool size. *Blood* *94*, 2605-12.

- Tolhuis, B., Palstra, R. J., Splinter, E., Grosveld, F., and de Laat, W. (2002). Looping and interaction between hypersensitive sites in the active beta-globin locus. *Mol Cell* 10, 1453-65.
- Trinh, L. A., Ferrini, R., Cobb, B. S., Weinmann, A. S., Hahm, K., Ernst, P., Garraway, I. P., Merckenschlager, M., and Smale, S. T. (2001). Down-regulation of TDT transcription in CD4(+)CD8(+) thymocytes by Ikaros proteins in direct competition with an Ets activator. *Genes Dev* 15, 1817-32.
- Tsai, F. Y., Keller, G., Kuo, F. C., Weiss, M., Chen, J., Rosenblatt, M., Alt, F. W., and Orkin, S. H. (1994). An early haematopoietic defect in mice lacking the transcription factor GATA-2. *Nature* 371, 221-6.
- Tsai, S. F., Martin, D. I., Zon, L. I., D'Andrea, A. D., Wong, G. G., and Orkin, S. H. (1989). Cloning of cDNA for the major DNA-binding protein of the erythroid lineage through expression in mammalian cells. *Nature* 339, 446-51.
- Tsai, S. F., Strauss, E., and Orkin, S. H. (1991). Functional analysis and in vivo footprinting implicate the erythroid transcription factor GATA-1 as a positive regulator of its own promoter. *Genes Dev* 5, 919-31.
- Tsang, A. P., Fujiwara, Y., Hom, D. B., and Orkin, S. H. (1998). Failure of megakaryopoiesis and arrested erythropoiesis in mice lacking the GATA-1 transcriptional cofactor FOG. *Genes Dev* 12, 1176-88.
- Tsang, A. P., Visvader, J. E., Turner, C. A., Fujiwara, Y., Yu, C., Weiss, M. J., Crossley, M., and Orkin, S. H. (1997). FOG, a multitype zinc finger protein, acts as a cofactor for transcription factor GATA-1 in erythroid and megakaryocytic differentiation. *Cell* 90, 109-19.
- Tuan, D. Y., Solomon, W. B., London, I. M., and Lee, D. P. (1989). An erythroid-specific, developmental-stage-independent enhancer far upstream of the human "beta-like globin" genes. *Proc Natl Acad Sci U S A* 86, 2554-8.
- Tyler, J. K., and Kadonaga, J. T. (1999). The "dark side" of chromatin remodeling: repressive effects on transcription. *Cell* 99, 443-6.
- Uchida, N., He, D., Frier, A. M., Reitsma, M., Sasaki, D., Chen, B., and Tsukamoto, A. (1997). The unexpected G0/G1 cell cycle status of mobilized hematopoietic stem cells from peripheral blood. *Blood* 89, 465-72.
- Vanin, E. F., Kaloss, M., Brosius, C., and Nienhuis, A. W. (1994). Characterization of replication-competent retroviruses from nonhuman primates with virus-induced T-cell lymphomas and observations regarding the mechanism of oncogenesis. *J Virol* 68, 4241-50.
- Varnum-Finney, B., Xu, L., Brashem-Stein, C., Nourigat, C., Flowers, D., Bakkour, S., Pear, W. S., and Bernstein, I. D. (2000). Pluripotent, cytokine-dependent, hematopoietic stem cells are immortalized by constitutive Notch1 signaling. *Nat Med* 6, 1278-81.

- Verfaillie, C. M., Pera, M. F., and Lansdorp, P. M. (2002). Stem cells: hype and reality. *Hematology (Am Soc Hematol Educ Program)*, 369-91.
- Verma, I. M., and Somia, N. (1997). Gene therapy -- promises, problems and prospects. *Nature* 389, 239-42.
- Vigna, E., and Naldini, L. (2000). Lentiviral vectors: excellent tools for experimental gene transfer and promising candidates for gene therapy. *J Gene Med* 2, 308-16.
- von Schwedler, U., Song, J., Aiken, C., and Trono, D. (1993). Vif is crucial for human immunodeficiency virus type 1 proviral DNA synthesis in infected cells. *J Virol* 67, 4945-55.
- Wadman, I. A., Osada, H., Grutz, G. G., Agulnick, A. D., Westphal, H., Forster, A., and Rabbitts, T. H. (1997). The LIM-only protein Lmo2 is a bridging molecule assembling an erythroid, DNA-binding complex which includes the TAL1, E47, GATA-1 and Ldb1/NLI proteins. *Embo J* 16, 3145-57.
- Wagers, A. J., Christensen, J. L., and Weissman, I. L. (2002). Cell fate determination from stem cells. *Gene Ther* 9, 606-12.
- Wall, L., Destroismaisons, N., Delvoye, N., and Guy, L. G. (1996). CAAT/enhancer-binding proteins are involved in beta-globin gene expression and are differentially expressed in murine erythroleukemia and K562 cells. *J Biol Chem* 271, 16477-84.
- Walters, M. C., Magis, W., Fiering, S., Eidemiller, J., Scalzo, D., Groudine, M., and Martin, D. I. (1996). Transcriptional enhancers act in cis to suppress position-effect variegation. *Genes Dev* 10, 185-95.
- Warren, A. J., Colledge, W. H., Carlton, M. B., Evans, M. J., Smith, A. J., and Rabbitts, T. H. (1994). The oncogenic cysteine-rich LIM domain protein rbtn2 is essential for erythroid development. *Cell* 78, 45-57.
- Wasylyk, B., Wasylyk, C., Matthes, H., Wintzerith, M., and Chambon, P. (1983). Transcription from the SV40 early-early and late-early overlapping promoters in the absence of DNA replication. *Embo J* 2, 1605-11.
- Watowich, S. S., Xie, X., Klingmuller, U., Kere, J., Lindlof, M., Berglund, S., and de la Chapelle, A. (1999). Erythropoietin receptor mutations associated with familial erythrocytosis cause hypersensitivity to erythropoietin in the heterozygous state. *Blood* 94, 2530-2.
- Weatherall, D. J. (2001). Phenotype-genotype relationships in monogenic disease: lessons from the thalassaemias. *Nat Rev Genet* 2, 245-55.
- Weatherall, D. J. (2001). Towards molecular medicine; reminiscences of the haemoglobin field, 1960-2000. *Br J Haematol* 115, 729-38.
- Weatherall, D. J., and Clegg, J. B. (1996). Thalassaemia--a global public health problem. *Nat Med* 2, 847-9.

- Wei, P., Garber, M. E., Fang, S. M., Fischer, W. H., and Jones, K. A. (1998). A novel CDK9-associated C-type cyclin interacts directly with HIV-1 Tat and mediates its high-affinity, loop-specific binding to TAR RNA. *Cell* 92, 451-62.
- Weiss, M. J., and Orkin, S. H. (1995). Transcription factor GATA-1 permits survival and maturation of erythroid precursors by preventing apoptosis. *Proc Natl Acad Sci U S A* 92, 9623-7.
- Weissman, I. L. (2000). Stem cells: units of development, units of regeneration, and units in evolution. *Cell* 100, 157-68.
- Wijgerde, M., Grosveld, F., and Fraser, P. (1995). Transcription complex stability and chromatin dynamics in vivo. *Nature* 377, 209-13.
- Wood, W. G. (1976). Haemoglobin synthesis during human fetal development. *Br Med Bull* 32, 282-7.
- Woods, N. B., Fahlman, C., Mikkola, H., Hamaguchi, I., Olsson, K., Zufferey, R., Jacobsen, S. E., Trono, D., and Karlsson, S. (2000). Lentiviral gene transfer into primary and secondary NOD/SCID repopulating cells. *Blood* 96, 3725-33.
- Yamada, Y., Warren, A. J., Dobson, C., Forster, A., Pannell, R., and Rabbitts, T. H. (1998). The T cell leukemia LIM protein Lmo2 is necessary for adult mouse hematopoiesis. *Proc Natl Acad Sci U S A* 95, 3890-5.
- Yoshida, C., Tokumasu, F., Hohmura, K. I., Bungert, J., Hayashi, N., Nagasawa, T., Engel, J. D., Yamamoto, M., Takeyasu, K., and Igarashi, K. (1999). Long range interaction of cis-DNA elements mediated by architectural transcription factor Bach1. *Genes Cells* 4, 643-55.
- Young, J. C., Bruno, E., Luens, K. M., Wu, S., Backer, M., and Murray, L. J. (1996). Thrombopoietin stimulates megakaryocytopoiesis, myelopoiesis, and expansion of CD34+ progenitor cells from single CD34+Thy-1+Lin- primitive progenitor cells. *Blood* 88, 1619-31.
- Yu, S. F., von Ruden, T., Kantoff, P. W., Garber, C., Seiberg, M., Ruther, U., Anderson, W. F., Wagner, E. F., and Gilboa, E. (1986). Self-inactivating retroviral vectors designed for transfer of whole genes into mammalian cells. *Proc Natl Acad Sci U S A* 83, 3194-8.
- Yusa, K., and Tsuruo, T. (1989). Reversal mechanism of multidrug resistance by verapamil: direct binding of verapamil to P-glycoprotein on specific sites and transport of verapamil outward across the plasma membrane of K562/ADM cells. *Cancer Res* 49, 5002-6.
- Zaitseva, M., Blauvelt, A., Lee, S., Lapham, C. K., Klaus-Kovtun, V., Mostowski, H., Manischewitz, J., and Golding, H. (1997). Expression and function of CCR5 and CXCR4 on human Langerhans cells and macrophages: implications for HIV primary infection. *Nat Med* 3, 1369-75.

- Zanjani, E. D., Almeida-Porada, G., Livingston, A. G., Flake, A. W., and Ogawa, M. (1998). Human bone marrow CD34⁻ cells engraft in vivo and undergo multilineage expression that includes giving rise to CD34⁺ cells. *Exp Hematol* 26, 353-60.
- Zennou, V., Petit, C., Guetard, D., Nerhbass, U., Montagnier, L., and Charneau, P. (2000). HIV-1 genome nuclear import is mediated by a central DNA flap. *Cell* 101, 173-85.
- Zhang, P., Behre, G., Pan, J., Iwama, A., Wara-Aswapati, N., Radomska, H. S., Auron, P. E., Tenen, D. G., and Sun, Z. (1999). Negative cross-talk between hematopoietic regulators: GATA proteins repress PU.1. *Proc Natl Acad Sci U S A* 96, 8705-10.
- Zhang, P., Zhang, X., Iwama, A., Yu, C., Smith, K. A., Mueller, B. U., Narravula, S., Torbett, B. E., Orkin, S. H., and Tenen, D. G. (2000). PU.1 inhibits GATA-1 function and erythroid differentiation by blocking GATA-1 DNA binding. *Blood* 96, 2641-8.
- Ziegler, B. L., Valtieri, M., Porada, G. A., De Maria, R., Muller, R., Masella, B., Gabbianelli, M., Casella, I., Pelosi, E., Bock, T., Zanjani, E. D., and Peschle, C. (1999). KDR receptor: a key marker defining hematopoietic stem cells. *Science* 285, 1553-8.
- Zufferey, R., Donello, J. E., Trono, D., and Hope, T. J. (1999). Woodchuck hepatitis virus posttranscriptional regulatory element enhances expression of transgenes delivered by retroviral vectors. *J Virol* 73, 2886-92.
- Zufferey, R., Dull, T., Mandel, R. J., Bukovsky, A., Quiroz, D., Naldini, L., and Trono, D. (1998). Self-inactivating lentivirus vector for safe and efficient in vivo gene delivery. *J Virol* 72, 9873-80.
- Zufferey, R., Nagy, D., Mandel, R. J., Naldini, L., and Trono, D. (1997). Multiply attenuated lentiviral vector achieves efficient gene delivery in vivo. *Nat Biotechnol* 15, 871-5.